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CORRECTION

In lines 31 and 34, page 320, Vol. 213, No. 1, March, 1955, read *0.08 N acetic acid* for *0.8 N acetic acid.*

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ALTERNATE CONVERSIONS OF GLYCEROL TO DIHYDROXYACETONE IN ACETOBACTER SUBOXYDANS*

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(Received for publication, September 13, 1954)

Acetobacter suboxydans produces dihydroxyacetone[1] from glycerol in high yields, either in the usual fermentation (1) or with resting cells (2, 3). DHA is oxidized further, but at a much lower rate. The present communication deals with the individual steps involved in the oxidation of glycerol by cell-free extracts of *A. suboxydans*, leading to the oxidation level of DHA. Details of the mechanism for dissimilation of DHA are given in the following paper (4).

Materials and Methods

Organism—*A. suboxydans*, ATCC 621, was used for the experiments. It was maintained on agar slants and transferred at weekly intervals. Transfers for inoculum were made into the growth medium to be used, as described below.

Reagents—ATP and DPN were commercial products obtained from the Pabst Laboratories. The ATP was found to be 94 per cent pure with respect to acid-hydrolyzable P. Of the total P content, 3.8 per cent was inorganic. 15 to 20 minute hydrolysis in 1 N acid was found to be necessary for complete release of the two P groups. TPN was obtained from

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[1] The following abbreviations are used throughout this paper: DHA, dihydroxyacetone; ATP, adenosinetriphosphate; CFE, cell-free extract; CoA, coenzyme A; DHA-P, DHA phosphate; DPN, diphosphopyridine nucleotide; GL-α-P, glycerol-α-phosphate; GL-β-P, glycerol-β-phosphate; P_a, alkali-hydrolyzable phosphate; P_i, inorganic phosphate; TCA, trichloroacetic acid; TPN, triphosphopyridine nucleotide; TTZ, triphenyltetrazolium chloride; TTZH, reduced TTZ (formazan); GL-P, glycerol phosphate; Tris, tris(hydroxymethyl)aminomethane.

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the Sigma Chemical Company. Glycerol-α-P was kindly furnished by Dr. B. L. Horecker, and was also isolated from a commercial mixture of the α and β phosphates (Na salt) obtained from General Biochemicals, Inc.

The separation of glycerol phosphates was performed with a 200 to 400 mesh strong base anion exchanger (Dowex 1), following the method of Khym and Cohn for separation of sugar phosphates (5); a 25 × 2.5 cm. column was washed with 2 N HCl and then washed acid-free with water. 3.9 gm. of the glycerol phosphates were freed of cations on a strong acid exchanger (Dowex 50) in the hydrogen form, and then neutralized with ammonia to pH 8.5 and placed on the Dowex 1 column. Elution was started with 0.025 M NH_4Cl-0.01 M $K_2B_4O_7$, and the borate concentration was reduced in a stepwise manner. This did not result in any appearance of GL-P in the eluate, as shown by spot tests on filter paper for organic P. Elution with 0.005 M HCl and 0.01 M HCl was likewise negative, but, when the eluent was changed to 0.02 M KCl-0.02 HCl, selective displacement of the isomers was achieved. This was evident from a comparison of the enzymatic assay results with the spot tests for organic P: only the α isomer was enzymatically oxidized (see "Results"). The fractions which, judged from paper chromatography, contained only GL-α-P were pooled and evaporated to dryness from the frozen state. The dry material was redissolved in water at pH 8.2. To this were added 0.5 volume of 1 M $BaCl_2$ and 3 volumes of acetone. Yield when dried, 340 mg. of Ba salt, which was found by paper chromatography to be free of P_i and glycerol-β-P.

Analytical Methods—For colorimetric determinations a Bausch and Lomb monochromatic colorimeter was employed. Oxidations with O_2 as the final electron acceptor were followed by standard Warburg manometric techniques. Other experiments employed commercial triphenyltetrazolium chloride *in vacuo* as the electron acceptor. 3 or more volumes of acetone were added at the end of the reaction to precipitate the protein and dissolve the reduced tetrazolium. After centrifugation of the solution, the color intensity was determined. A standard curve relating micromoles of TTZH per 10 ml. of volume to optical density was prepared from samples of TTZ^+ that had been enzymatically reduced with a large excess of substrate.

Other colorimetric determinations may be carried out on reaction mixtures containing TTZH. Trichloroacetic acid will precipitate the unchanged TTZ, and one centrifugation thus removes both TTZ^+, TTZH, and protein material. Possible interference in sugar and P tests that involve reductions is thus eliminated.

For P_i and organic P determinations, the Fiske-Subbarow method was used (6) on a 2.5 ml. total volume scale. Alkali-hydrolyzable P plus P_i was determined as above after 20 minutes hydrolysis with 1 N NaOH at room temperature, and P_a obtained by difference.

Paper Chromatography—Whatman filter paper No. 1 was cut into sheets of 6 × 22 inches and washed with 1 N HCl. Before the final wash with deionized water, the papers were treated with a 0.02 per cent Versene solution. The solvent system used routinely was ethyl acetate-acetic acid-water in the ratio 3:3:1 (7). Descending chromatograms were run at room temperature. The solvent front reached the bottom of the paper in 8 to 10 hours, and was usually allowed to proceed onto a filter paper roll taped to the chromatogram. This device allowed a more uniform and faster flow off the paper than could be obtained by drainage. After 15 to 20 hours the papers were removed and dried before a fan. The phosphate esters were located on the chromatograms by the method of Hanes and Isherwood (8).

Preparation of Cells—The major part of the cell material was produced by aerating batches of 200 liters of inoculated medium in a modified conical-cylindrical steam-jacketed kettle. To 200 liters of water were added 20 pounds of glycerol, 4 pounds of Difco yeast extract, 2 pounds of KH_2PO_4, and 0.3 pound of glucose. The mixture was boiled 30 minutes, with 5 to 10 pounds pressure in the jacket. While the medium was cooling, some air was admitted through the aeration system to keep a slight positive pressure in the kettle. Dispersion of the air was accomplished through a system of twelve spherical spargers connected radially to an inlet tube in the center of the kettle. The air was preheated, with the heater thermostatically controlled, so as to keep the medium at 30° during growth. The medium was inoculated with 10 liters of broth of the same composition containing an actively growing *A. suboxydans* culture. After 40 to 60 hours, the cells were harvested in a Sharples supercentrifuge, resuspended in a Waring blendor into 4 liters of 0.05 M phosphate buffer at pH 6, and shaken at room temperature for 1 hour to deplete them of oxidizable substrates. The cells were then recentrifuged and suspended twice in distilled water, and, after the last centrifugation, were freeze-dried *in vacuo*. Yields varied from 50 to 80 gm., dry weight (0.25 to 0.4 gm. per liter). Purity of the cultures was checked by microscopic and plate methods.

Preparation of Cell-Free Extracts—These were obtained by grinding with alumina (Alcoa A-301) (9). The soluble preparations were usually made from 2.5 gm. of freeze-dried cells and were stored at −10°. In order to remove nucleotides such as CoA, ATP, and DPN, this crude extract was treated for use in certain experiments with Dowex 1, following a procedure by Novelli and Schmetz (10).

Results

Phosphorylative and Non-Phosphorylative Oxidation of Glycerol—Resting cells oxidized glycerol most rapidly at pH 6 to 7. This was also the pH range that permitted the greatest yield of DHA during fermentation (1).

The oxidation rate diminished about 30 per cent at pH 8.0, and nearly disappeared at pH 8.5 (Table I). A corresponding decrease was observed for the cell-free extract. However, at pH 8.5 it was possible to obtain rapid oxidation when ATP was added. ATP was usually without influence at pH 6.0, although some stimulation was noted in a few experiments. A phosphorylative oxidation of glycerol at the alkaline pH was also indi-

TABLE I

Phosphorylative and Non-Phosphorylative Oxidation of Glycerol

ATP	Q_{O_2}					
	pH 6.0			pH 8.5		
	Whole cells	CFE*		Whole cells	CFE*	
	O_2	O_2	TTZ	O_2	O_2	TTZ
−	10	23	0	0.6	0	0
+	10	23	0	0.6	12	9

* Cell-free extract.

Each Warburg vessel contained 50 μmoles of malate buffer at pH 6.0 or 100 μmoles of Tris at pH 8.5, 5 μmoles of $MgCl_2$, 0.2 ml. of 20 per cent KOH in the center well. The ATP concentration was 1 μmole per ml. wherever used. The whole cell experiments contained in addition 40 μmoles of glycerol, 10 mg. of cells (dry weight), and water to 3 ml. 3 μmoles of DPN were added, together with ATP at pH 8.5. The CFE experiments with O_2 had 30 μmoles of glycerol, 20 μmoles of NaF, and 1 ml. of CFE in a total volume of 2 ml.; atmosphere = air. The tubes in the TTZ experiments were incubated *in vacuo* with 5 μmoles of glycerol, 50 μmoles of Tris, 10 μmoles of NaF, 2.5 μmoles of $MgCl_2$, 5 μmoles of TTZ, 0.1 μmole of DPN, and 0.1 ml. of CFE in a total volume of 1 ml. Q_{O_2} for whole cells, microatoms of O_2 per mg. of dry cells per hour; for CFE, microatoms of O_2, or micromoles of TTZ per ml. of CFE (27 mg., dry weight, 18 mg. of protein) per hour. Time intervals used to compute Q_{O_2} (usually 20 to 40 minutes) were sufficient to permit conversion of up to 20 per cent of the added glycerol to DHA. Temperature, 30°.

cated when TTZ was the final electron acceptor in cell-free extracts. The elucidation of this mechanism was then sought with the latter system.

Requirement for DPN, ATP, and Mg—Oxidation of glycerol in alkaline medium takes place only in the presence of DPN. In Fig. 1, *A*, the maximal activity was achieved with 10^{-4} M DPN. In most experiments a slight drop in oxidation rate was observed when the DPN was increased 10-fold, although in others the curve reached a plateau above 5×10^{-4} M. The reduced activity at high concentrations may possibly be due to a binding of Mg^{++} which is also needed for the oxidation. Experiments reported below confirm this explanation. TPN was not active as an elec-

tron acceptor with this dehydrogenase. The endogenous oxidation rate in this and the other TTZ experiments was practically zero.

ATP was not active in catalytic amounts. When its concentration was increased beyond a certain threshold value, it stimulated the oxidation

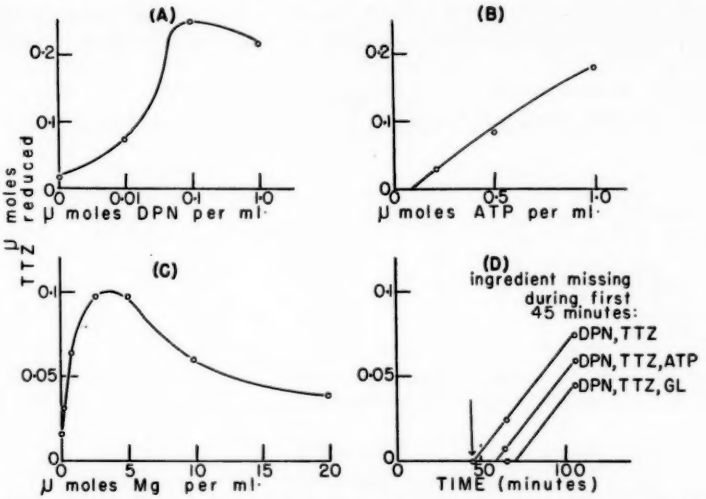

FIG. 1. The lag period in glycerol oxidation, and the requirement for DPN, ATP, and Mg^{++}. *A*, the reaction mixture contained 5 μmoles of GL, 25 μmoles of Tris at pH 8, 5 μmoles of TTZ, 10 μmoles of F^-, 2.5 μmoles of Mg^{++}, 1.0 μmole of ATP, 0.1 ml. of Dowex-treated CFE, graded levels of DPN, and water to 1.0 ml. Time, 60 minutes. *B*, conditions as in *A*, except that 0.1 μmole of DPN was used throughout, and the ATP concentration varied as indicated. *C*, conditions as in *B*, except that 1 μmole of ATP was used, and the Mg^{++} concentration varied as indicated. *D*, conditions as in *A*, except that 2.5 μmoles of Mg^{++} were used. The reaction mixture was incubated without DPN, TTZ, or the indicated missing ingredient for 45 minutes, then was added as shown. The values for TTZH production in the respective experiments are not strictly comparable, since they were performed at different times.

(Fig. 1, *B*), and was most effective in concentrations approaching that of the substrate (complete data are not shown). For routine work, 1 μmole of ATP was used per ml., since at higher levels it was oxidized itself, or stimulated some endogenous oxidation.

Mg^{++} was obligatory in the reaction mixture (Fig. 1, *C*), particularly with Dowex-treated extracts. Apparently Dowex 1 removed much of the Mg^{++} present. This may have been due to adsorption or to binding by the nucleotides removed by the anion exchanger. However, when the

Mg^{++} concentration exceeded 2.5×10^{-3} M, a gradual decrease in the rate of oxidation was observed.

Lag Period in Glycerol Oxidation—In the previous experiments, the oxidation was stopped when sufficient TTZH was produced to give useful readings in the colorimeter. When the development of color was followed with time, there was an initial period during which no detectable oxidation took place. After the oxidation began, it proceeded with uniform velocity

TABLE II

Chromatography of Phosphate Esters from Glycerol-ATP Reaction

Sample	Position constants observed*		
A. Reaction mixture = ATP + CFE	0.11	0.46 (Faint)	
B. (A) + glycerol	0.11	0.33 "	0.83
C. " + GL-α-P	0.11	0.36 "	0.86
D. GL-α-P			0.87
E. ATP	0.07		
F. Adenylic acid		0.34	

* The position constant is the distance traveled by the compound divided by the distance traveled by P_i. For components of the reaction mixture, see the text.

TABLE III

Formation of Triose P from GL-α-P in A. suboxydans

Time	TTZH	Triose P
min.	*μmoles*	*μmole*
0	0.00	0.00
60	0.56	0.29
120	1.60	0.54

6 μmoles of GL-α-P were incubated with 0.2 ml. of CFE, 5 μmoles of TTZ, 0.1 μmole of DPN, 50 μmoles of Tris buffer at pH 8.0, 10 μmoles of F^-, and H_2O to 1 ml. Temperature, 30°. Triose P was measured as alkali-labile P (P_a).

for a considerable time. Reducing the amount of ATP prolonged the lag period.

These facts suggested that glycerol must be phosphorylated before it can be oxidized, and that the lag period corresponded to the time necessary for sufficient phosphorylation to take place to allow visible oxidation. It was therefore thought that the lag period might be removed by preincubating the CFE anaerobically with all the components except TTZ and DPN. The addition of these compounds could then be expected to permit immediate oxidation, whereas omission of glycerol or ATP during the preincubation period should result in a new lag period. This was found to

be true, as shown in Fig. 1, *D*. When GL-α-P was then substituted for glycerol and ATP, no lag period was observed, and the oxidation proceeded at the same rate in the absence of ATP as for glycerol in the presence of ATP. GL-β-P was inert.

Chromatographic Identification of GL-α-P from Reaction Mixture—The preceding experiments pointed strongly toward the existence in the reaction mixture of a phosphorylated derivative of glycerol, presumably GL-α-P. A chromatographic analysis of the reaction mixture was accordingly undertaken. 10 μmoles of ATP were incubated with a large excess of glycerol in the presence of 25 μmoles of $MgCl_2$, 100 μmoles of NaF, 250 μmoles of

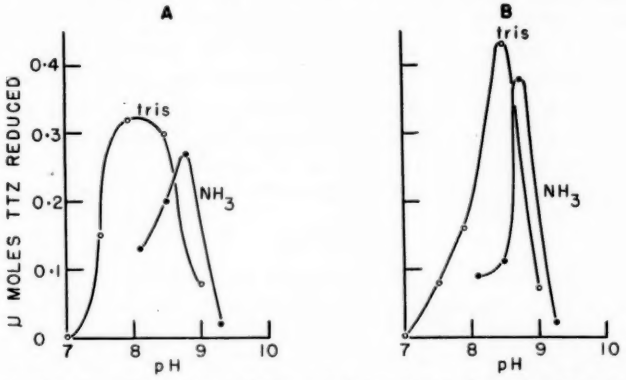

FIG. 2. pH effect upon the oxidation of glycerol and glycerol-α-phosphate. *A*, 0.1 ml. of CFE was incubated with 2.5 μmoles of GL, 25 μmoles of Tris or NH_3, 1.25 μmoles of Mg^{++}, 5 μmoles of F^-, 0.5 μmole of ATP, 2.5 μmoles of TTZ, 0.05 μmole of DPN, and water to 0.5 ml.; time, 30 minutes; temperature, 30°. *B*, conditions as in *A*, with ATP and Mg^{++} omitted and 1 μmole of GL-α-P replacing GL.

Tris buffer (pH 8), and 1 ml. of CFE; volume, 10 ml. A blank was included containing no glycerol. The mixture was allowed to react *in vacuo* at 30° until a definite red color developed (about 30 minutes) in an aliquot of the reaction mixture to which had been added TTZ and DPN. TCA was then added to a concentration of 3 per cent, and the protein centrifuged off at high speed. The supernatant fluid was extracted five times with ether, adjusted to pH 4, and the liquid lyophilized. The residue was dissolved in 0.2 ml. of H_2O and applied on the chromatogram, in quantities of 6 μl. per spot. The results of the separation are shown in Table II.

It is apparent that a new phosphate compound had appeared in the reaction mixture in the presence of glycerol, and that its position on the chromatogram approximated that of GL-α-P.

Oxidation of GL-α-P; Mg^{++}-DPN Interaction—When GL-α-P was oxi-

dized, formation of triose P could be observed. This is shown in Table III. Mg^{++} was not required for this oxidation; however, when a series of Mg^{++} and DPN levels was examined, it was found that the higher the Mg^{++}, the higher must be the DPN concentration to maintain a given oxidation rate.

pH Optimum for Oxidation of Glycerol and GL-α-P—Fig. 2, *A* shows that the maximal oxidation rate of glycerol extends from pH 7.8 to 8.5, dropping rather sharply in more acid or alkaline media. When GL-α-P was the substrate, the peak narrowed to produce a sharp optimum at about pH 8.5 (Fig. 2, *B*). When NH_4OH was used as a buffer, a shift in the optimum to pH 8.8 occurred.

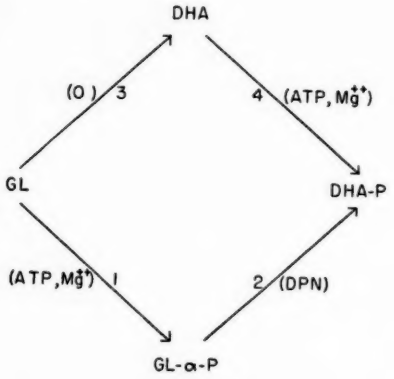

DIAGRAM 1

DISCUSSION

The data presented show the existence of a glycerokinase, a glycerol dehydrogenase, and a GL-α-P dehydrogenase in the soluble cell-free extract of *A. suboxydans*. As shown in the following paper (4), the extracts also contain a DHA kinase, and the accompanying two routes, given in Diagram 1, from glycerol to DHA-P are therefore possible. The presence in the extracts of a very active triose phosphate isomerase (4) did not allow the identification of the first isomer produced in Reaction 2. Based on steric considerations (11), however, DHA phosphate would be expected to be the primary product. A need for DPN with the glycerol dehydrogenase was not detected. This oxidation may possibly be coupled directly to a cytochrome. This might also furnish an explanation why TTZ is not reduced by the glycerol dehydrogenase, since intervention of a flavoprotein would presumably be necessary for the latter reaction.

Reaction 2, coupled to Reaction 1, was employed to measure the rate of phosphorylation of glycerol when the concentrations of glycerol, ATP, and Mg were varied. At higher concentrations of Mg^{++} (above 2.5 μmoles per ml.; Fig. 1, *C*), the decrease in rate of formation of TTZH was pronounced. This may be explained simply in terms of formation of a Mg-ATP complex (12), which would limit the formation of the postulated intermediary ATP-Mg-enzyme complex and, accordingly, the GL-α-P formation. In addition, the Mg^{++}-DPN interaction noted above would contribute to lower oxidation at high concentrations of Mg^{++}. Such complex formation of Mg^{++} by nucleotides may be responsible for its removal from cell-free extracts by anion exchange resins.

A partial stimulation of oxygen consumption by ATP has been observed in a few preparations at pH 6.0. The failure to reproduce this is in all probability due to the generally observed absence of kinase activity below pH 7 (Fig. 2). Maintenance of a slightly acid environment during fermentation may thus operate to prevent the functioning of both the glycerol and DHA (4) kinases, and effectively to inhibit the further utilization of DHA under these conditions, as discussed in a previous communication (3).

SUMMARY

The oxidation of glycerol by cell-free extracts of *Acetobacter suboxydans* has been found to proceed by two pathways: one, which is apparently independent of ATP and DPN, proceeds at pH 6 to the formation of dihydroxyacetone; the other, with a pH optimum about 8.5, requires the participation of ATP and Mg^{++} in a kinase reaction, to yield glycerol-α-phosphate. The latter is then oxidized by a DPN-dependent dehydrogenase to form dihydroxyacetone phosphate.

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OXIDATION OF DIHYDROXYACETONE VIA THE PENTOSE CYCLE IN ACETOBACTER SUBOXYDANS*

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Acetobacter suboxydans has for many years been regarded as an organism possessing broad but limited ability to attack sugars and other polyhydroxy compounds (1). More recently, it has become apparent that the organism possesses a number of enzymes for more extensive dissimilation of substrates such as glucose, glycerol, and sorbitol (2). On the other hand, di- and tricarboxylic acid cycle activities appear to be absent in this organism (3), and several important components of the normal Embden-Meyerhof pathway are also lacking.

In the present work attention has been focused on dihydroxyacetone[1] in an attempt to trace its fate in the oxidative machinery of the cell. In order to study individual reactions and to allow the free access of phosphorylated intermediates to enzyme surfaces, the organization of the whole cells was in nearly all experiments sacrificed and soluble cell-free extracts were employed.

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[1] The following abbreviations are used throughout this paper: ATP, adenosinetriphosphate; DHA, dihydroxyacetone; DHA-P, dihydroxyacetone phosphate; DPN, diphosphopyridine nucleotide; F-6-P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; G, glucose; G-6-P, glucose-6-phosphate; GLA, glyceraldehyde; GL, glycerol; GLA-P, glyceraldehyde-3-phosphate; GL-α-P, glycerol-α-phosphate; P_a, alkali-hydrolyzable phosphate; P_i, inorganic phosphate; 6-PGA, 6-phosphogluconic acid; R, ribose; R-5-P, ribose-5-phosphate; SH, sedoheptulose; SH-P, sedoheptulose phosphate; TCA, trichloroacetic acid; TPN, triphosphopyridine nucleotide; TTZ, triphenyltetrazolium chloride; CFE, cell-free extract; PC, position constant; Tris, tris(hydroxymethyl)aminomethane.

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Materials and Methods

These were as described in the preceding paper (4), with the following additions.

Reagents—Ribose and ribose-5-phosphate, Ba salt, were obtained from the Schwarz Laboratories, Inc. The latter possessed 97 per cent of the theoretical organic P content, and its ribose equivalent was 100 per cent, as measured by the orcinol test (5). However, paper chromatography revealed three phosphate spots, and it seems likely that other ribose (or ribulose) phosphates may have been present in this preparation. Glucose-6-phosphate was obtained from the Sigma Chemical Company as the Ba salt. Its total P content was 78 per cent of the theoretical value, and inorganic P comprised 3 per cent of the total. DHA was obtained from the Krishell Laboratories, Inc. Fructose-1,6-diphosphate was obtained from the Schwarz Laboratories, Inc., as the Mg salt. It was about 68 per cent pure as measured by the resorcinol assay (5). After concentration by ion exchange chromatography and precipitation as the Ba salt, as suggested by Hers *et al.* (6), the product possessed a ratio of organic P to fructose of 2.01:1, although the organic phosphate content was only 80 per cent of theory. Dihydroxyacetone phosphate was prepared from FDP through the action of a partially purified yeast aldolase (described below) in the presence of hydrazine. The triose P, separated from unchanged FDP by ion exchange chromatography, contained only about 10 per cent glyceraldehyde-3-P, due to the presence of some triose P isomerase in the aldolase preparation. Further separation of the triose phosphates was not carried out, since it was found that *A. suboxydans* cell-free extracts also contain this isomerase and thus establish an equilibrium between the two isomers. The hydrazone of DHA-P was decomposed by treatment with benzaldehyde, and the DHA-P was precipitated as the Ba salt after extraction of excess benzaldehyde with ether. 6-Phosphogluconic acid was prepared according to Seegmiller and Horecker (7) from G-6-P. Although the 6-GPA gave only one phosphate spot when chromatographed on paper, either with ethanol-acetate-water or ethyl acetate-acetic acid-water as solvents, analysis revealed only 82 per cent of the theoretical P content. Of this, 4 per cent was P_i.

Analytical Methods—A Beckman model B spectrophotometer was used for absorption spectrum analysis. For P determination, the method of Martin and Doty (8) was in some instances superior to the one of Fiske and Subbarow (9). The time curves for enzymatic release of P_i from phosphorylated metabolites, for example, were studied by this method: 0.1 ml. aliquots of the enzyme reaction mixture were at intervals pipetted into the two-phase system of 1 ml. of isobutyl alcohol-benzene, 0.4 ml. of

water, and 0.2 ml. of silicotungstate reagent. The protein material precipitated and accumulated at the interphase. At the end of the experiment ammonium molybdate was added, and the 8 ml. tubes were shaken vigorously for 15 seconds. This brought the phosphomolybdate into the organic phase. A 0.5 ml. aliquot was removed and diluted to 1.9 ml. with acid alcohol. Stannous chloride was then used to produce the molybdenum blue color. The color intensity from a given amount of P_i was about 3.5 times as high in the Martin-Doty method as in the Fiske-Subbarow determination. It has recently been pointed out that the development of the molybdenum blue directly in trichloroacetic acid extracts may lead to considerable error due to various interferences, and that the phosphomolybdate should be extracted into an organic phase before reduction (10).

GLA-P was determined as alkali-labile P before and after oxidation by iodine; the 3-phosphoglycerate produced was stable to alkali (11).

Ribose P was determined by the orcinol test at 660 mμ, following the method outlined by LePage (5). Hexose and heptulose derivatives absorbed light in the range 500 to 600 mμ, but with lower intensities than pentose. At 660 mμ, the relative absorptions given by equimolar concentrations of ribose, fructose, and glucose were 1.0, 0.10, and 0.05, respectively. Simultaneous assay for sedoheptulose and hexose phosphates was carried out with the cysteine-sulfuric acid test (12). Absorption curves for various compounds reacting with the orcinol and cysteine reagents are given in an accompanying paper (13).

Aldolase activity was measured according to Sibley and Lehninger (14) and from the production of P_a, in both cases with FDP as substrate. When the two methods were used simultaneously, there was good agreement between the assay values. Hydrazine was ineffective in trapping the triose phosphates in a 1:1 ratio in the present work. Invariably an absorption spectrum was produced with a peak at 540 mμ, whereas the DHA and glyceraldehyde derivatives showed absorption maxima at 530 and 440 mμ, respectively.

Enzyme Preparation—Yeast aldolase was concentrated from brewers' yeast through alcohol and acetone precipitations of Lebedev juice, according to a method given by Warburg (15).

Paper Chromatography—Procedures were the same as in the preceding communication (4). In general, phosphate esters in the reaction mixtures were identified by comparison with authentic samples added to reaction blanks. The effects of other compounds in the concentrated reaction mixtures on the mobility of the esters were thus compensated for. A list of position constants, relating the mobility of a particular compound to that of P_i, is given in Table I.

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Results

Oxidizability of DHA—The oxidation of DHA proceeded only after an initial lag period. This was observed first for resting cells, as shown in Fig. 1, where the rate of oxygen consumption was consistently lower than the rate of glycerol (GL) oxidation beyond the DHA stage. This deficiency was largely removed by preincubating the organisms with DHA. Adaptive enzyme formation was suggested by this behavior, but was ruled out by the fact that this stimulatory effect was produced not only by DHA

TABLE I

Position Constants and R_F Values of Some Phosphate Esters

Compound	PC*	R_F	Solvent system
P_i	1.00	0.45	Ethyl acetate-acetic acid-water (3:3:1)
Pyrophosphate	0.58		
GL-α-P	0.93		
DHA-P	0.94		
R-5-P	0.70		
G-6-P	0.37		
F-6-P	0.57		
FDP	0.40		
6-PGA	0.44		
AMP	0.35		
ATP	0.19		
G-6-P		0.19	80% ethanol, 0.8% AcOH-KOAc, final pH 5.5
F-6-P		0.23	
6-PGA		0.12	

* PC = the position constant = the distance traveled by the compound divided by the distance traveled by P_i.

but also by a number of other substrates oxidizable by *A. suboxydans*, including glycerol, glucose, sorbitol, sorbose, and ethanol. If on the other hand the cells were preincubated with buffer alone, or acetate or citrate, which could not be oxidized, no stimulation was observed. This indicated that DHA underwent some endergonic conversion prior to its oxidation, possibly involving phosphorylation.

Experiments with cell-free extracts confirmed this suspicion. The oxidation, which was catalyzed by Mg^{++} and diphosphopyridine nucleotide and required adenosinetriphosphate in substrate amounts, was preceded by a lag period of 25 minutes. When the concentration of DHA, ATP, or the enzyme was lowered, the lag period was prolonged, but preincubation of these three components plus Mg^{++} eliminated the lag altogether; *i.e.*, oxidation began immediately upon addition of DPN and triphenyltetrazolium chloride.

The data were quantitatively and qualitatively almost identical to those obtained for the phosphorylative oxidation of glycerol (4), and suggested strongly the action of a DHA kinase to form DHA-P prior to oxidation. When DHA-P was tested, it was oxidized rapidly in the absence of ATP. This oxidation had its peak at a somewhat lower pH than the glycerol-α-P oxidation (4), namely at pH 8.1, and was not as sensitive to alkali; considerable activity remained at pH 9.5. However, the most important difference lay in the fact that Mg^{++} was obligatory for DHA-P oxidation.

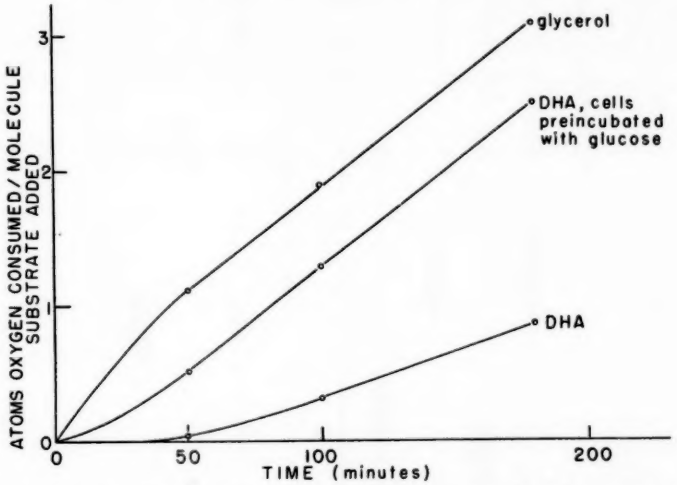

FIG. 1. Oxidation of DHA by resting *A. suboxydans* cells. For the preincubation, 70 mg. of dry cells were suspended in 1400 μmoles of glucose, 7.5 μmoles of DPN, 500 μmoles of P_i, 100 μmoles of $MgCl_2$, and H_2O to 10 ml. at pH 6.0. The reaction mixture was shaken for 90 minutes, and the cells were centrifuged and washed repeatedly. They were then placed in Warburg flasks in 3 ml. of medium of the same composition, except that the substrate was 40 μmoles of DHA. The center well contained 0.2 ml. of 20 per cent KOH; temperature, 30°.

Chromatographic Identification of DHA-P from Reaction Mixture—The procedure was the same as with the glycerol-α-P (4), with DHA as substrate instead of glycerol. A new spot appeared, corresponding to that produced by authentic DHA-P, as shown in Fig. 2. When the reaction mixture containing the DHA-P was treated with 1 N alkali for 20 minutes, subsequent chromatography of the neutralized sample showed that all the DHA-P had disappeared. DHA-P could be easily distinguished from GL-α-P, which possessed a similar R_F value, by the much more rapid hydrolysis of DHA-P on the paper; an intense blue spot developed from DHA-P with only slight heating.

Non-Oxidative Enzymatic Reactions Involving DHA-P: Aldolase and Triose P Isomerase—Since DHA-P in other organisms is a member of the aldolase and triose P isomerase equilibria, experiments were designed to test for the presence of these two enzymes.

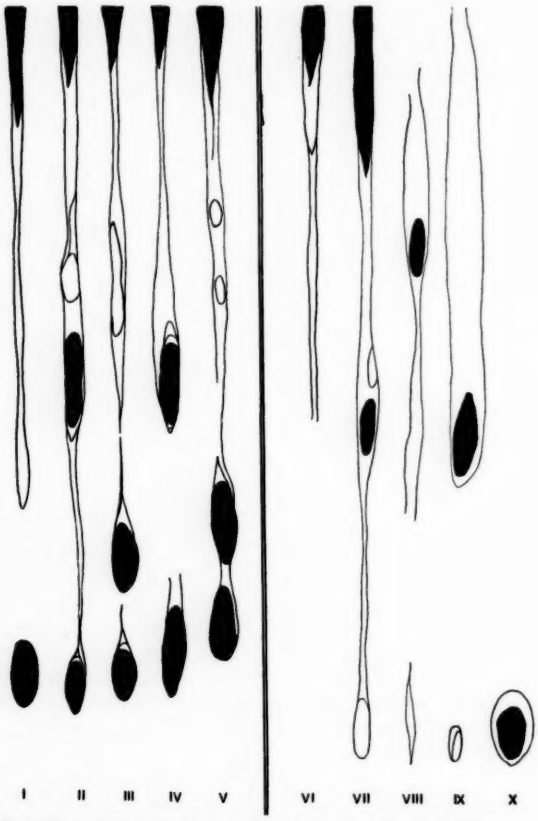

FIG. 2. Chromatography of phosphate esters from DHA-ATP interaction. The reaction mixture contained 0.5 ml. of CFE, 125 μmoles of Tris buffer at pH 8.0, 50 μmoles of F^-, 12.5 μmoles of Mg^{++}, 50 μmoles of ATP, and H_2O to 20 ml. DHA, when added, comprised 125 μmoles. After incubation for 30 minutes, TCA was added to make a 3 per cent solution and the mixture was centrifuged. *I*, reaction mixture, no DHA, 10 μl. were applied to filter paper; *II*, (*I*) alkali-hydrolyzed, 30 μl.; *III*, (*I*) + DHA, 10 μl.; *IV*, (*III*), alkali-hydrolyzed, 30 μl.; *V*, (*I*), with DHA-P added after TCA, 10 μl.; *VI*, ATP only, 0.1 μmole; *VII*, ATP only, 0.3 μmole, alkali-hydrolyzed; *VIII*, adenylic acid only, 0.2 μmole; *IX*, pyrophosphate only, 0.2 μmole; *X*, P_i only, 0.1 μmole.

With samples of commercial FDP, extracts of *A. suboxydans* showed rapid aldolase action according to the Sibley and Lehninger test (14). The absorption spectrum of the colored derivative formed corresponded very closely to that from DHA. The results were confirmed several times with purified FDP. This also allowed formation of triose P to be followed in terms of P_a. When an aliquot of the reaction mixture was oxidized by iodine, and P_a determined afterward, it could be shown that only small amounts of GLA-P were formed (Table II).

Pentose-P Breakdown—Another potential non-oxidative reaction involving triose P is the system producing triose P from R-5-P. It has been shown recently for other systems (12, 16) that, in addition to triose P, there are also formed SH and hexose phosphates through the action of

TABLE II

Demonstration of Aldolase and Isomerase in A. suboxydans Extracts

Sample	$P_a + P_i$	P_i
	μmoles	*μmole*
1. Complete mixture	1.80	0.40
2. " iodine-oxidized	1.75	
3. (1), FDP added after TCA	0.70	0.60
4. (2), " " " "	0.81	
5. (1), no FDP	0.40	0.33
6. (2), " "	0.42	

The system contained 0.2 ml. of CFE, 5 μmoles of FDP, 50 μmoles of Tris buffer at pH 8.0, and 15 μmoles of F^-, incubated at 37° *in vacuo* for 20 minutes; volume, 1.5 ml. 1.3 ml. of 10 per cent TCA were added, neutralized, and centrifuged. The iodine oxidation was carried out as previously described (11).

transketolase and transaldolase. When R-5-P was incubated without DPN *in vacuo* with the cell-free extract and assays were made at intervals for triose P, SH, pentose, and hexose, the results were as shown in Fig. 3. The cysteine-sulfuric acid test afforded both a qualitative and quantitative identification of SH (12).

Rôle of Mg^{++} in DHA-P Oxidation—It was noted above that Mg^{++} was required for oxidation of DHA-P. This was also found to be true with FDP as substrate, as might be expected in view of the presence of aldolase and isomerase in the extract. G-6-P was oxidized rapidly by the cell-free extracts, but here Mg^{++} was without effect. Mg^{++} in other systems is required for the specific release of the phosphate group from the 1 position of FDP (17, 18). These observations on the Mg effect, together with the data on the R-5-P conversion, suggested that the cyclic mechanism described recently by Horecker (19) might prevail in DHA oxidation in *A. suboxydans*. In the following sections and accompanying

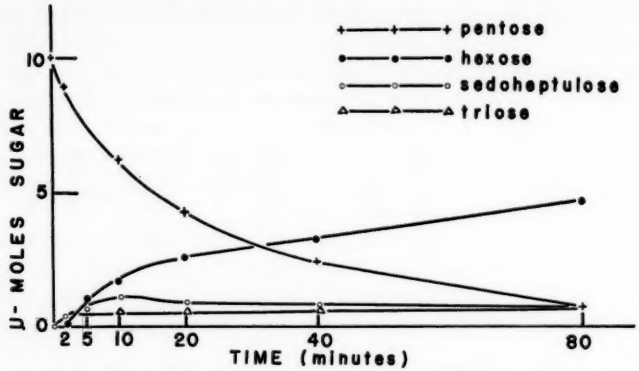

FIG. 3. Non-oxidative breakdown of ribose-5-P by cell-free extracts. The tubes were incubated *in vacuo* with 10 μmoles of R-5-P, 100 μmoles of Tris at pH 8.0, 20 μmoles of F^-, 0.15 ml. of CFE, and water to 2 ml. The reaction was stopped by adding 1.6 ml. of 10 per cent TCA. Pentose, hexose, and SH were assayed as described in the text, with R-5-P, glucose-6-P, and sedoheptulosan as standard. Mutual interferences in the determinations necessitated only minor corrections in the early stages (up to 20 minutes), although after longer incubation periods the corrections in the orcinol pentose assay due to hexose were appreciable, and the pentose values were therefore of reduced significance. Triose was measured as alkali-hydrolyzable P (triose P).

papers (13, 20) it will be referred to as the *pentose cycle* because of the key position of pentose P in the accompanying diagram. The conversion of R-5-P to hexose P in liver and in spinach extracts involves the intermediate formation of sedoheptulose P (SH-P) and probably tetrose P in the

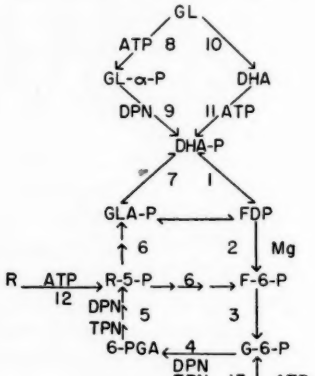

DIAGRAM 1

following postulated fashion (18):

(1) $4C_5P \xleftarrow{TK,\ PI} 2C_7P + 2C_3P \xrightarrow{TA} 2C_4P + 2C_6P$

(2) $2C_5P + 2C_4P \xleftrightarrow{TK} 2C_3P + 2C_6P$

(3) $2C_3P \xleftrightarrow{TI,\ A} C_6P_2$

Sum, $6C_5P \xleftrightarrow{} 4C_6P + C_6P_2$

where TK = transketolase, TA = transaldolase, A = aldolase, PI = pentose P isomerase, and TI = triose P isomerase. In the presence of Mg the total reaction is

$$6C_5P \rightarrow 5C_6P + P_i$$

Evidence for Reactions within Pentose Cycle

Reactions 11, 7, 1, 2, 3 (ATP + DHA → F-6-P ↔ G-6-P)—The presence in the cell-free extract of the isomerase-aldolase system was noted above, tested with FDP as substrate. With DHA as the starting material, it was possible, in the presence of ATP and Mg^{++}, to show the formation of fructose in amounts (as measured by the resorcinol test) approaching the theoretical value based on the ATP present, assuming activity of ADP in this system, either *per se* or through a myokinase. The reactions could also be followed through observation of the inorganic P released (Fig. 4). Under non-oxidative conditions, the P_i release reached a plateau sharply at the theoretical value of 1 μmole of P_i per μmole of ATP, the other phosphate group presumably remaining as fructose-6-phosphate (F-6-P). The observed reaction rates for formation of fructose and P_i were similar. Under oxidative conditions (TTZ and DPN) a further, slower release of P_i was observed, as would be expected if the pentose cycle were operating; meanwhile hexose accumulation dropped to about one-tenth of the amount formed non-oxidatively. Fluoride at a concentration of 10^{-2} M or greater was found to be of value in reducing ATPase activity (to about 30 per cent of the value in the absence of fluoride). The same fluoride concentration allowed only slight hydrolysis of FDP or F-6-P.

Reaction 2 (FDP → F-6-P)—It was found that FDP released P_i only in the presence of Mg^{++}. As shown in Fig. 5, *A*, this liberation of P_i ceased at a concentration of 1 μmole of P_i per μmole of FDP. The resulting hexose monophosphate showed the stability toward acid hydrolysis that would be expected for a hexose ester with the P group in the 6 position. On a paper chromatogram the products of Reaction 2 produced two spots characteristic of an authentic mixture of glucose-6-P and fructose-6-P, with the former predominating. Like the corresponding enzyme in liver (21, 22), this was an alkaline phosphatase (Fig. 5, *B*). The optimal Mg^{++}

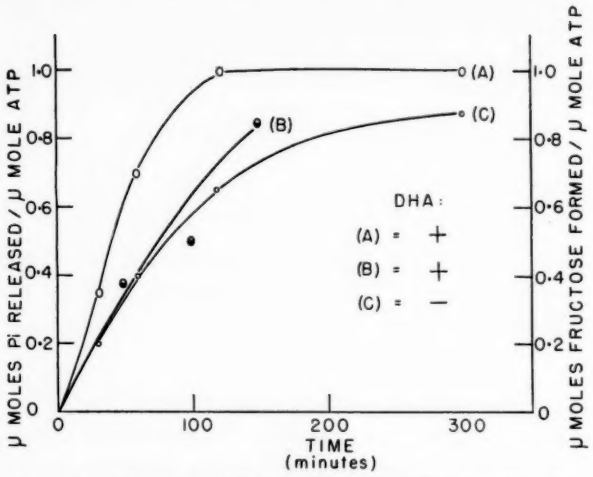

FIG. 4. Formation of hexose and P_i from DHA and ATP in *A. suboxydans*. Curves A and C, P_i release; Curve B, fructose formation (following experiment performed separately). 0.10 ml. of CFE was incubated with 1.0 μmole of ATP, 5 μmoles of DHA, 10 μmoles of F^-, 25 μmoles of Tris buffer at pH 8.0, 2.5 μmoles of Mg^{++}, and H_2O to 1.0 ml.; temperature, 30°.

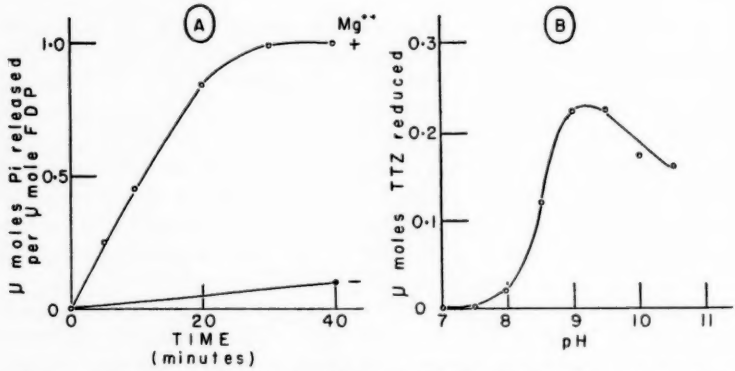

FIG. 5. FDP phosphatase of *A. suboxydans* (FDP → F-6-P + P_i). *A*, 2.8 μmoles of FDP were incubated with 0.2 ml. of CFE in the presence of 100 μmoles of Tris at pH 8.5, 200 μmoles of F^-, 5 μmoles of Mg^{++}, and water to 2.0 ml. Aliquots were removed at intervals for P_i assay; temperature, 30°. *B*, pH optimum for action of the enzyme. Conditions were as in *A*, except that glycine buffer was used from pH 9.5 to 10.5, and Tris from pH 7.0 to 9.5. Time, 20 minutes.

concentration was about 2.5 μmoles per ml., with the enzymatic activity slowly decreasing at higher concentrations.

Reaction 3 (*F-6-P → G-6-P*)—A very active phosphohexose isomerase was present in *A. suboxydans*. Incubation of G-6-P with the cell-free extract led to formation of a material that reacted in the resorcinol test for fructose. Chromatograms of the reaction mixture again revealed both F-6-P and G-6-P. These compounds were equally readily oxidized, indicating the relatively greater speed of the interconversion.

Reactions 4 and 5 (*G-6-P → 6-PGA → R-5-P + CO_2*)—When the oxidation of G-6-P by the cell-free extract was followed manometrically, there was an initial lag in the rate of CO_2 production, compared to O_2 uptake. G-6-P and F-6-P were also oxidized in the presence of TTZ and DPN. Analysis of the reaction products by the orcinol test showed formation of

TABLE III

Oxidation of 6-Phosphogluconic Acid by A. suboxydans

	0 min.	30 min.	55 min.	90 min.
	μmole	*μmole*	*μmole*	*μmole*
TTZH	0	0.19	0.39	0.75
Pentose	0	0.12	0.12	0.14

5 μmoles of 6-PGA were incubated with 0.1 ml. of CFE and 50 μmoles of Tris buffer at pH 8.0, 50 μmoles of F^-, 7.5 μmoles of TTZ^+, 0.05 μmole of DPN, and water to 0.5 ml. Temperature, 30°.

a compound with maximal absorption at 660 mμ, unless the oxidation was extensive (above 3 μmoles of TTZH produced per μmole of substrate). Chromatography of the products of G-6-P oxidation revealed zones corresponding to 6-PGA and R-5-P. (For the identification of 6-PGA, the ethyl acetate-water system in Table I was employed.) Identification of ribulose-5-phosphate or other pentose phosphates was not sought. 6-PGA was oxidized by the enzyme preparation, in the presence of TTZ and DPN. Formation of pentose with time was followed, as shown in Table III. The low level of pentose maintained is reasonable in view of the rapid transformation of R-5-P (Table IV).

The unexpected activity of both DPN and TPN in the G-6-P and 6-PGA dehydrogenases has not yet been clarified. Under the conditions of the present experiments, where cycling of the component sugars occurred, TPN was about twice as effective as DPN for G-6-P oxidation. In the oxidation of 6-PGA, DPN was more active than TPN at concentrations of 0.01 μmole per ml., but the reverse was true at 0.1 μmole per ml. Studies are in progress to fractionate these enzymes further and determine their

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exact pyridine nucleotide requirements. A similar lack of specificity for these compounds has been reported in the corresponding dehydrogenases of *Pseudomonas fluorescens* (23).

Reaction 6 (R-5-P → SH-P, GLA-P, and F-6-P)—R-5-P is oxidized rapidly in the cell-free extracts with DPN and TTZ. It also disappears under non-oxidative conditions, as noted previously. The immediate appearance of SH and triose, and the subsequent formation of hexose, strongly indicates the operation of transketolase-transaldolase systems, in addition to pentose P isomerase. SH was also formed non-oxidatively from F-6-P, with about the same ratio (1:10) of SH to hexose monophosphate as in the conversion from R-5-P. The F-6-P → SH-P conversion might involve a transketolation of F-6-P to some endogenous acceptor of

TABLE IV

Relative Reaction Rates within Pentose Cycle

Reaction No.	Q, μmoles per hr. per ml. of CFE	Ml. of CFE per ml.	Remarks
1	42	0.04	Measured at 37°
2	42	0.1	
3			Fast compared to Reaction 4
4	28	0.1	With DPN
5	8	0.1	" "
6	215	0.1	
7			Fast compared to Reaction 1

Temperature, 30°. Other conditions were as described for the respective reactions (Table II, Table III, Fig. 3, and Fig. 5).

active glycolaldehyde, with the tetrose residue interacting with F-6-P to produce SH-P and triose P (24). The equilibrium of the pentose-hexose conversion lies far to the right, as is evident from Fig. 3. Starting with R-5-P, addition of Mg^{++} to the reaction mixture significantly decreased the yield of triose P, as would be expected during a further conversion of FDP to F-6-P.

Paper chromatography of the ribolytic reaction mixture in comparison with authentic samples of R-5-P, F-6-P, G-6-P, and DHA-P revealed the presence of each of these compounds, as well as SH. SH was identified on the chromatogram by the specific orcinol spray (25). Upon enzymatic hydrolysis of the R-5-P reaction mixture with alkaline phosphatase and subsequent chromatography, all the phosphate appeared as P_i, and the position of the SH changed from R_F 0.15 to 0.29 (ethyl acetate-acetic acid-water). This suggested that SH also exists in the organism as a phosphate ester.

Reaction Rates in Pentose Cycle—Although specific attention was not given to the kinetic relationships of the cycle reactions in the present work, the experiments performed provide some information regarding the relative rates. The conversion rates listed in Table IV refer to the amounts of reactant appearing or disappearing in an initial time interval that allowed only a small fraction of the substrate to be utilized. The slow utilization of 6-PGA, as compared to the preceding dehydrogenation of G-6-P and the very rapid subsequent disappearance of R-5-P, is noteworthy. The rapid removal of R-5-P observed here raises the question whether the pentose levels recorded in Table III should be somewhat lower. It is possible that the pentose assay results may have been spuriously high due to the presence of hexose and sedoheptulose, since these sugars interfere to some extent in the orcinol pentose assay (13).

Reactions Leading into Pentose Cycle

Reactions 8, 9 (*GL* → *GL-α-P* ↔ *DHA-P*), *Reactions 10, 11* (*GL* → *DHA* → *DHA-P*)—These reactions are described in the accompanying paper (4).

Reaction 12 (*R* → *R-5-P*)—The presence of a ribokinase is so far indicated only by the fact that ribose itself is not oxidized unless ATP is present.

Reaction 13 (*G* → *G-6-P*)—A partially purified glucokinase, which still contains phosphohexose isomerase, has been obtained from the cell-free extract. With glucose as substrate, the hexose phosphates could be isolated as the Ba salts in good yields, and in a ratio corresponding to the establishment of the phosphohexose isomerase equilibrium (3 G-6-P:1 F-6-P).

DISCUSSION

The soluble cell-free extracts of *A. suboxydans* contain the spectrum of enzymes necessary for the operation of the pentose cycle. This cycle furnishes a mechanism for the complete oxidation to CO_2 and water of any of the cycle members, or of the metabolites connected to the cycle by preliminary phosphorylation or oxidation reactions. Upon one complete turn of the cycle, 6 molecules of hexose monophosphate yield 6 molecules of CO_2 and 1 molecule of P_i, consume 6 molecules of oxygen, and return to the starting point as 5 molecules of hexose monophosphate.

The pentose cycle in particular furnishes a pathway for the oxidation of DHA-P in this organism, and, although not necessarily the only one, it is the first yet to be uncovered. A direct oxidation of triose P seems not to play a significant rôle. In cell-free extracts, no oxidation of DHA-P took place in the absence of Mg, indicating a lack of the traditionally Mg-independent triose P dehydrogenase. Furthermore, pyruvate appears not

to be on the route of glycerol and DHA oxidation, since only small amounts of acetic acid can be detected upon extensive oxidation of glycerol by resting cells (3). Lactate oxidation with resting cells and pyruvate oxidation in cell-free extracts lead to nearly theoretical yields of acetate (26, 27). The observed oxidation of DHA by resting cells on the other hand fits the operation of the pentose cycle. The O_2 uptake and the CO_2 output are approximately linear with time, and the respiratory quotient is 1.0, corresponding to oxidation to CO_2 and water (see also King and Cheldelin (28)). The fact that the O_2 consumption by resting cells ceases when it reaches two-thirds of the amount calculated for complete combustion of DHA remains to be explained, although it does not necessarily conflict with the operation of the cycle if it may be assumed that the remainder of the sugar is assimilated. Extensive assimilation of other substrates has been observed by resting yeast cells (29, 30). However, it seems less likely that assimilation could also account for the failure of the CFE to oxidize glycerol beyond 5 atoms of O per molecule,[2] and the possibility remains that additional pathways of glycerol metabolism may operate in *A. suboxydans*.

The pentose cycle opens a pathway for oxidation, not only of glycerol and DHA in this organism, but also of glucose and ribose. The possible connection of sorbose to this scheme is not yet clear. Here also additional oxidative routes cannot be excluded, since it is known, for example, that glucose is converted to gluconic and ketogluconic acids in large yields in fermentations with this organism.

Although *A. suboxydans* is the only microorganism in which the pentose cycle has thus far been demonstrated *in toto*, this mechanism may be of general significance in many microorganisms. Key reactions of the cycle, such as the "hexose monophosphate shunt" and the pentose-sedoheptulose conversion, have been observed for other bacteria and yeast (23, 31). This establishes another biochemical link between microbial species and the plant and animal kingdoms in which the pentose cycle was first discovered (12, 19).

SUMMARY

Oxidation of dihydroxyacetone by cell-free extracts of *Acetobacter suboxydans* depends first upon an ATP-linked kinase reaction. The dihydroxyacetone phosphate formed is then converted, through isomerase and aldolase equilibria, to hexose diphosphate. Dephosphorylation, isomerization, and oxidation of glucose-6-phosphate ensue, to produce phosphogluconic acid and ribose-5-phosphate, in the presence of either DPN or TPN. A non-oxidative, ribolytic series of reactions can also be demon-

[2] Kitos, P. A., King, T. E., and Cheldelin, V. H., unpublished observations.

strated, which leads to the early appearance of sedoheptulose and triose, and the subsequent formation of hexose. This indicates the presence of transaldolase-transketolase systems, as well as a pentose phosphate isomerase. The reactions of Horecker's pentose cycle thus appear to be operative *in toto* in this organism, with an expected and observed respiratory quotient of 1.0 for the oxidation of dihydroxyacetone.

Other substrates, such as glucose, ribose, and glycerol, also are capable of entering the pentose cycle, through preliminary kinase or oxidative reactions.

Addendum—Since the submission of this manuscript, it has been found possible (Kitos, P. A., King, T. E., and Cheldelin, V. H., unpublished results) to remove DPN, TPN, Mg^{++}, and cocarboxylase from the cell-free extracts by adsorption on Dowex 50. In these treated extracts, TPN is specifically required for reactivation of G-6-P dehydrogenase, and DPN for reactivation of 6-PGA dehydrogenase (Reactions 4 and 5 in Diagram 1).

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CARBOHYDRATE OXIDATION BY THE WHEAT SMUT FUNGUS, TILLETIA CARIES*

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Extensive genetic studies (1) and limited nutritional investigations (2, 3) have been accomplished for the wheat smut organism, *Tilletia caries*, but information is lacking concerning its metabolism and enzyme systems. A more systematic approach to control might be realized by a fuller understanding of the nutrition and metabolism of the smut fungus.

Studies designed to elucidate the pathway of carbohydrate metabolism show that maltose, starch, and glucose are rapidly oxidized by resting cells and the phosphorylated sugars by cell-free extracts. The results with the phosphorylated esters suggest the operation of the Horecker pentose cycle, which has been demonstrated in other species. Thus this mechanism of metabolizing carbohydrate is recognized in another organism. The presence of hexokinase and aldolase has also been shown in the cell-free extracts.

EXPERIMENTAL

Growth of Organism—The organism used for these studies, *Tilletia caries*, D-11-7, was obtained from Dr. C. S. Holton of the State College of Washington.

The fungus was grown in 5 or 12 liter round bottom flasks with forced aeration, or in 500 ml. Erlenmeyer flasks on a rotary shaker. The medium consisted of 5 gm. of tryptone, 10 gm. of glucose, and 1 gm. of yeast extract per liter. Maximal cell yields were obtained by aseptically blending the inoculum suspension in a Waring blendor prior to inoculation. Use of this method increased the number of mycelium particles, and during growth the clones changed from a few large balls to many smaller ones. The improved aerobic conditions obtained for individual balls of mycelium probably accounted for the higher yields. 3 gm. of dry cells were obtained

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per liter of medium in 24 hours at 20°. A drop in pH from 7.5 to 6.8 occurred in the 24 hour period.

The cells were harvested by filtration on a Büchner funnel and washed twice with deionized water. They were then used for resting cell experiments or for preparation of acetone powders.

Cell-Free Preparations—Acetone powders were prepared by blending the cells in a Waring blendor with 10 volumes of acetone (−10°) for 1 minute and filtering the resulting powder. The acetone powder was blended again in 5 volumes of acetone for 30 seconds, recovered by filtration, dried *in vacuo*, and stored in a deep freeze. The preparation was stable for several months, although the oxidative activity decreased slowly.

Cell-free extracts were prepared as needed from the acetone powder. 0.5 gm. was homogenized with 15 ml. of 0.02 M phosphate or Tris[1] buffer at pH 7.5 in a Virtis homogenizer or a Potter-Elvehjem glass homogenizer. The extract was then centrifuged for 1 hour at 10,000 × *g* and the supernatant liquid used for the experiments, after dialysis at 0° for 2 to 3 hours against 10 liters of 0.02 M Tris buffer at pH 7.5. Removal of pyridine nucleotides was accomplished, when desired, by dialysis against Dowex 1 (chloride form) suspended in the outer chamber.

Cell-free extracts were also prepared by grinding 0.5 gm. of acetone powder with 7 gm. of Alumina A-301 and 2 ml. of 0.02 M Tris buffer. The preparation was then extracted for 30 minutes with 15 ml. of 0.02 M Tris buffer at pH 7.5 with frequent stirring. The enzymes for the studies to be noted were obtained by centrifugation at 10,000 × *g* for 1 hour. All preparations and centrifugations were performed at 0°. No significant differences in activity were noted among the methods for preparing the cell-free extracts.

Methods and Materials

Oxygen uptake or carbon dioxide evolution was measured manometrically with conventional Warburg equipment. A Beckman model B spectrophotometer was used for the spectrophotometric analysis. Protein was measured by a modified biuret method of Weichselbaum (4). Crystalline egg albumin was used for the standard. Hexokinase was measured by the method of Colowick and Kalckar (5). Aldolase was determined by the method of Bard and Gunsalus (6). Inorganic phosphorus was measured by the method of Ernster *et al.* (7). Pentose was estimated by the orcinol

[1] The following abbreviations are used throughout this paper: DPN, diphosphopyridine nucleotide; F-6-P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; P_i, inorganic phosphate; 6-PGA, 6-phosphogluconic acid; R-5-P, ribose-5-phosphate; TPN, triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosinetriphosphate.

method (8), fructose by Roe's resorcinol method (8), and sedoheptulose and hexose by the sulfuric acid-cysteine method (9). Triose was measured by alkali-labile phosphate or by its production after aldolase action (6). Conventional ascending paper chromatographic methods were used. The paper was washed by the method of Mortimer (10). The solvent systems employed were those of Bandurski and Axelrod (11) or Hauge *et al.* (12). The molybdate spray of Bandurski *et al.* (11) was used for the detection of phosphate esters. Phosphate esters from the reaction mixtures were identified by cochromatography with authentic samples. Corrections for absorption by interfering compounds were applied when necessary, particularly when assaying pentose in the presence of larger amounts of hexose or sedoheptulose (13). The following chemicals were all commercial preparations: Glucose-6-phosphate (Sigma), fructose-6-phosphate (Nutritional Biochemicals), Armour coenzyme concentrate (Armour), and ribose-5-phosphate (Schwarz). G-6-P was oxidized with bromine for the preparation of 6-phosphogluconic acid (14). Phosphate analysis indicated 80 per cent organic P. Chromatography gave only a spot for 6-PGA and inorganic P. Fructose-1,6-diphosphate was a commercial preparation which was purified by column chromatography (12). The purity of this ester was checked by the resorcinol test and the amount of organic P present. Paper chromatography indicated only FDP, P_i, and fructose to be present; by the resorcinol test it was found to contain 100 per cent of the theoretical amount of fructose. 85 per cent of the phosphate present was organic P.

Diphosphopyridine nucleotide (Pabst) and triphosphopyridine nucleotide (Sigma "80") were commercial preparations.

N-Methylphenazine sulfate was kindly furnished by Dr. R. H. Burris of the University of Wisconsin.

Results

Resting cells oxidized glucose, maltose, and starch, as shown in Fig. 1. Maltose was oxidized more rapidly than any of the other carbohydrates, and the hexose phosphates only to a limited extent. The addition of more glucose to the reaction vessel resulted in continued oxidation, indicating disappearance of substrate after the utilization of 4 μatoms of oxygen per μmole. Fructose and sucrose were also oxidized by the resting cells, although to a more limited extent than the carbohydrates previously mentioned.

The presence of hexokinase is shown in Fig. 2. The respective phosphate esters formed in the reactions were demonstrated by paper chromatography. Based on the ATP present, the CO_2 yield (from bicarbonate) was about 50 per cent of theory for complete conversion of glucose to G-6-P.

Aldolase was present in the cell-free preparation, as shown in Fig. 3. The pH optimum of the *Tilletia* enzyme was 8.0.

Cell-free extracts oxidized glucose-1-phosphate, G-6-P, FDP, F-6-P, 6-PGA, and R-5-P, as shown in Table I. Of the tricarboxylic acid cycle members tried, only succinate was oxidized, and it only very feebly. Maximal oxygen uptake obtained was 5 μatoms of oxygen per micromole

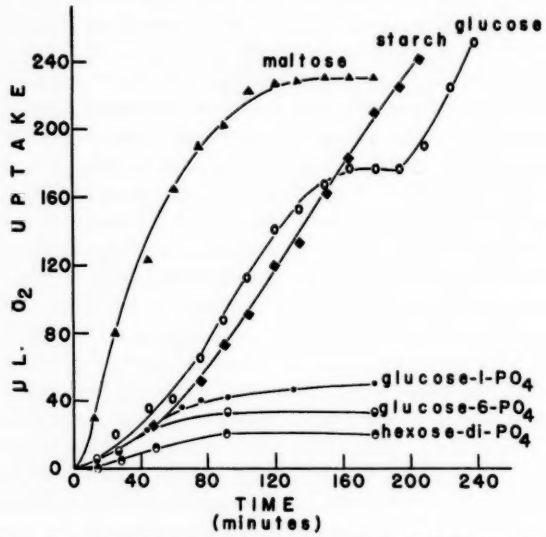

FIG. 1. Oxidation of carbohydrates by resting cells of *Tilletia*. Each Warburg vessel contained 1.0 ml. of cell suspension (50 mg., dry weight); 5 μmoles of substrate (except starch, which was added as 150 μmoles of glucose equivalent in a soluble-starch preparation); 0.3 ml. of 20 per cent KOH in a center well; 100 μmoles of phosphate buffer, pH 7.5. Total volume 3.0 ml.; temperature, 28°. Gas, air. 5 μmoles of glucose were added initially and again at 200 minutes.

of G-6-P, with a respiratory quotient of 1. The oxidation of this compound was also more rapid than that of the other phosphate esters. The oxidations were followed with *N*-methylphenazine sulfate as the electron carrier since it was found that the preparations were quite active with this compound (more so than with cytochrome *c*). Fluoride also had a stimulatory effect, apparently because of inhibition of a phosphatase. The pattern of oxidation of the phosphate esters suggested the operation in *T. caries* of the pentose cycle which is present in animals (15), higher plants (11, 15), microorganisms (12), and insects (13). For this reason a systematic study was undertaken to demonstrate the presence of the following

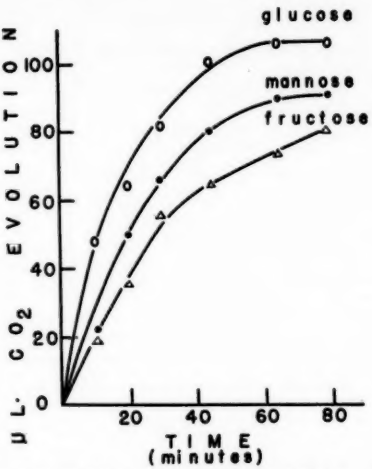

FIG. 2. Hexokinase in a cell-free preparation of *Tilletia*. Each Warburg vessel contained 10 μmoles of $MgCl_2$; 60 μmoles of NaF; 20 μmoles of glucose (mannose or fructose); 10 μmoles of ATP; 4 mg. of protein; 16 μmoles of bicarbonate. Total volume, 2.3 ml.; temperature, 28°. Gas, 95 per cent nitrogen-5 per cent carbon dioxide.

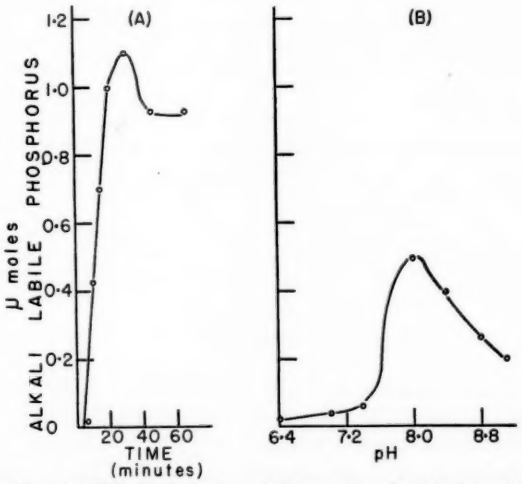

FIG. 3. Aldolase in *Tilletia* extracts. *A*, formation of triose from hexose diphosphate with time. *B*, pH optimal curve for aldolase; incubated for 15 minutes. Incubation mixture: 3.5 μmoles of fructose-1,6-diphosphate; 150 μmoles of Veronal buffer at pH 7.2; 140 μmoles of hydrazine hydrochloride at pH 7.2; cell-free extract (3 mg. of protein). Total volume, 2.25 ml.; temperature, 37°.

TABLE I

Oxidation of Hexose Phosphates by Cell-Free Preparation of T. caries

Substrate	μatoms oxygen per μmole substrate added initially					
	7 min.	15 min.	22 min.	30 min.	52 min.	68 min.
G-6-P	0.97	1.6	1.9	2.2	2.6	3.0
G-1-P	0.48	0.81	0.89	1.1	1.2	1.2
FDP	0.42	0.71	0.90	1.1	1.3	1.4
F-6-P	0.08	0.45	0.52	0.61	0.8	0.9
R-5-P		0.18	0.22	0.43	0.7	0.9
6-PGA				0.18	0.45	0.58

Each Warburg vessel contained 7 mg. of protein, 4 μmoles of substrate, 60 μmoles of Tris buffer at pH 7.4, 100 μmoles of nicotinamide, 1 mg. of Armour coenzyme concentrate, 0.2 mg. of *N*-methylphenazine sulfate. Total volume, 3.0 ml.; temperature, 28°. Gas, air.

series of reactions, suggested by Horecker (15), in the accompanying Diagram 1.

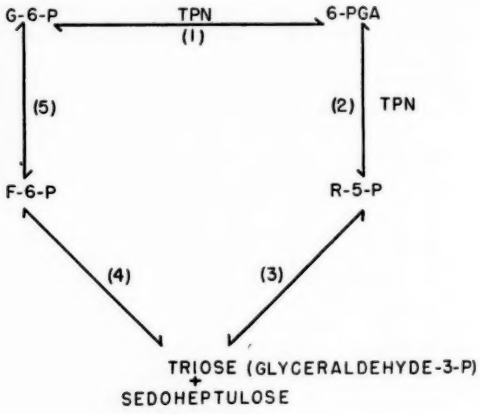

DIAGRAM 1

Reaction 1—The presence of G-6-P dehydrogenase was demonstrated by following TPN reduction at 340 mμ, as illustrated in Fig. 4, *A*. The addition of G-1-P also resulted in a reduction of TPN, although at a slower rate, indicating the presence of phosphoglucomutase. TPNH was reoxidized in this system by acetaldehyde. DPN was only slightly active. The oxidation of G-6-P with O_2 as an electron acceptor was also TPN-dependent, with only slight reaction occurring in the presence of DPN. The formation of 6-PGA from G-6-P was demonstrated by cochromatography on paper.

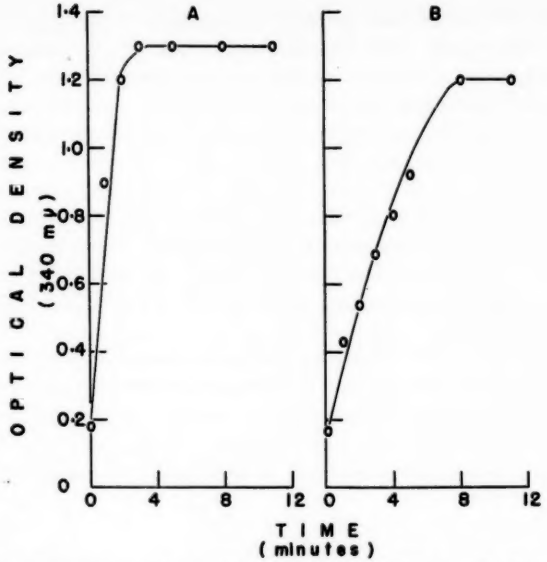

FIG. 4. *A*, glucose-6-phosphate dehydrogenase of *Tilletia*. Each cuvette contained 3 mg. of protein, 0.8 μmole of G-6-P, 30 μmoles of Tris buffer at pH 7.5, 0.32 μmole of TPN. Suitable blanks were included in which enzyme, substrate, or TPN was omitted; temperature, 25°. *B*, 6-phosphogluconic dehydrogenase. Reaction mixture the same as *A*, except that the substrate was 6-PGA, pH 7.5.

TABLE II

Formation of Sedoheptulose, Triose, and Hexose from Ribose-5-phosphate Anaerobically

Compound	μmoles formed					
	0 min.	2 min.	5 min.	15 min.	45 min.	60 min.
Pentose	10	5.9	3.9	3.0	3.2	3.1
Sedoheptulose	0	1.9	2.5	1.0	1.3	1.3
Hexose	0	0.5	1.0	1.4	1.2	1.4
Triose	0	1.2	1.5	1.5	1.1	1.5
Total*	10	9.8	9.8	7.0	7.1	7.5

* Micromoles of carbon divided by 5.

The incubation mixture consisted of cell-free extract prepared with a Potter-Elvehjem glass homogenizer and containing 10 mg. of protein; 10 μmoles of ribose-5-phosphate; 200 μmoles of Tris buffer, pH 7.5; temperature, 30°. Total volume, 1C ml. 1 ml. aliquots were removed at the stated intervals and pipetted into 1 ml. of 10 per cent trichloroacetic acid.

Reaction 2—The cell-free extracts contained 6-PGA dehydrogenase, as shown in Fig. 4, *B*. The formation of R-5-P was demonstrated by cochromatography on paper. Sedoheptulose, pentose, and fructose were also detected colorimetrically from 6-PGA: 0.74 μmole of sedoheptulose, 0.45 μmole of pentose, and 0.19 μmole of fructose were obtained from 4 μmoles of 6-PGA in 120 minutes.

Reactions 3, 4, and 5—Evidence for the occurrence of transketolase and transaldolase (16, 17) was obtained by following the formation of sedoheptulose, triose, and hexose from R-5-P anaerobically. The results are shown in Table II. That the hexoses formed were F-6-P and G-6-P was demonstrated by cochromatographing a reaction mixture in which R-5-P was the substrate.

Reaction 5—The presence of phosphohexose isomerase was demonstrated by following the reverse reaction and measuring the fructose formed. The production of 2 μmoles of fructose in 40 minutes from 8 μmoles of G-6-P was observed with the cell-free preparation. In the presence of TPN and fructose-6-phosphate, the formation of sedoheptulose and pentose was observed.

DISCUSSION

T. caries is able to oxidize the carbohydrates that might be expected to occur in the wheat plant. It is of interest that only the non-phosphorylated carbohydrates are oxidized by the whole cells to a considerable extent and that hexokinase is present, thereby enabling the organism to oxidize carbohydrates by known mechanisms involving the phosphate esters. Nutritional studies have shown that *T. caries* can grow on a simplified medium containing only asparagine as a source of nitrogen (2, 3); however, growth on such a purified medium is much slower and less extensive than that obtained in these experiments.

These studies have extended the presence of the pentose cycle to another group of organisms quite different from those which have previously been shown to contain the cycle. The implications are discussed in the following paper (13).

The authors are indebted to Dr. S. M. Dietz and Dr. J. R. Hardison of the Department of Botany and Plant Pathology, Oregon State College, for their valuable suggestions.

SUMMARY

The oxidative behavior of the wheat smut organism, *Tilletia caries*, has been examined. This has been facilitated by development of a liquid medium which permits luxuriant growth in a 24 hour period. Acetone

extracts were prepared which contain hexokinase, aldolase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, and the enzymes of the pentose cycle.

Oxidation of glucose, maltose, and starch and limited oxidation of fructose and sucrose have been shown, but little activity was found when various Krebs cycle acids were used as substrates.

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OXIDATION OF CARBOHYDRATES BY THE PEA APHID, MACROSIPHUM PISI (KLTB.)*

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(Received for publication, September 13, 1954)

The nutrition and metabolism of aphids are of interest to biochemists, since these important pests depend upon leaf cell sap for their nutrition and excrete large amounts of carbohydrate and amino acids (1, 2). This suggests that they may have some special nutritional requirements (3), and that their carbohydrate metabolism may possess unusual features.

The evidence obtained to date indicates the absence of a hexokinase system, but shows that hexose phosphates are readily utilized. The organism contains the enzymes necessary for oxidation of these compounds by way of the Horecker pentose cycle (4). Thus this cyclic mechanism has been demonstrated in another type of organism differing from higher animals, higher plants, bacteria, or the wheat smut fungus, in which the cycle has been previously established (4–7).

EXPERIMENTAL

Pea aphids, *M. pisi* (Kltb.), were grown on young pea or horse-bean plants in the greenhouse, and harvested as needed for enzyme preparations. Freshly collected aphids were blended in a Waring blendor for 1 minute with 10 volumes of acetone (−10°) and filtered, and the powder was again blended with 5 volumes of acetone. The acetone powder after filtration was dried *in vacuo* and stored at −10°. This preparation was stable for 4 to 6 months with respect to the enzyme systems studied. Cell-free preparations were obtained in a manner similar to that employed in the preceding communication (7). Centrifugation was for 1 hour at 20,000 × *g*.

Materials and methods were the same as in the preceding paper, except that tetrazolium (TTZ)[1] reductions were employed as in the report of

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[1] The following abbreviations are used throughout this paper: CoA, coenzyme A; DPN, diphosphopyridine nucleotide; F-6-P, fructose-6-phosphate; FDP, fructose-1,-6-diphosphate; FMN, flavin mononucleotide; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; 6-PGA, 6-phosphogluconic acid; R-5-P, ribose-5-phosphate;

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Hauge *et al.* (6). The following chemicals were all commercial preparations: α-ketoglutaric acid (Krishell Laboratories); Armour coenzyme concentrate (containing about 25 γ of CoA per mg., 7 per cent DPN, 4 per cent TPN, 0.25 per cent pyridoxal phosphate plus pyridoxamine phos-

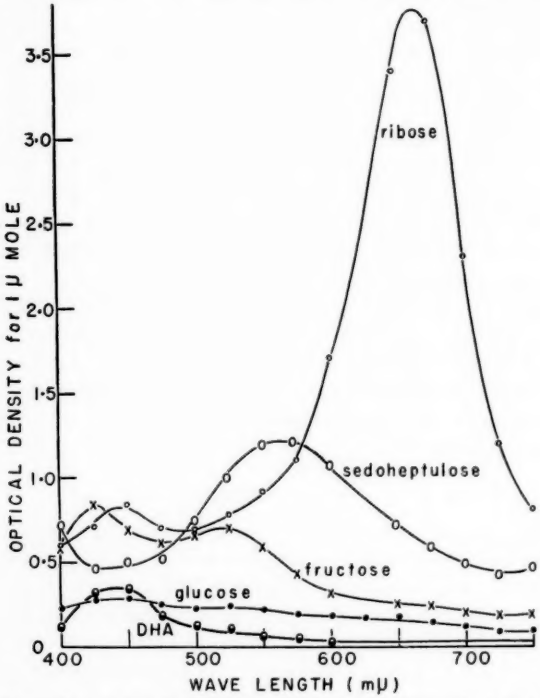

FIG. 1. Absorption curves for various compounds in the orcinol test. 1 μmole of each compound was used, except that 0.1 μmole of ribose was used and corrected to 1 μmole in order to obtain a suitable plot. 3 ml. of 1 per cent orcinol in 0.1 per cent $FeCl_3$ in concentrated HCl were added to 3 ml. of the compound to be tested. The solution was heated at 100° for 30 minutes, cooled, and the readings made within 40 minutes.

phate); pyocyanine (F. Hoffmann-La Roche and Company, Ltd.); and flavin mononucleotide (FMN) (Sigma).

In the previous papers (6, 7) it was noted that certain corrections were necessary because of interfering absorptions by sugars other than those being measured in the orcinol and cysteine-sulfuric acid methods. Figs. 1 and 2 illustrate the interferences obtained and the absorption curves for

SH, sedoheptulose; TPN, triphosphopyridine nucleotide; TTZ, triphenyl tetrazolium hydrochloride.

the sugars that might be present in the reaction mixtures. Table I gives the molar extinction coefficients calculated from the absorptions of the sugars at the wave-lengths used in the experiments. Some interferences may

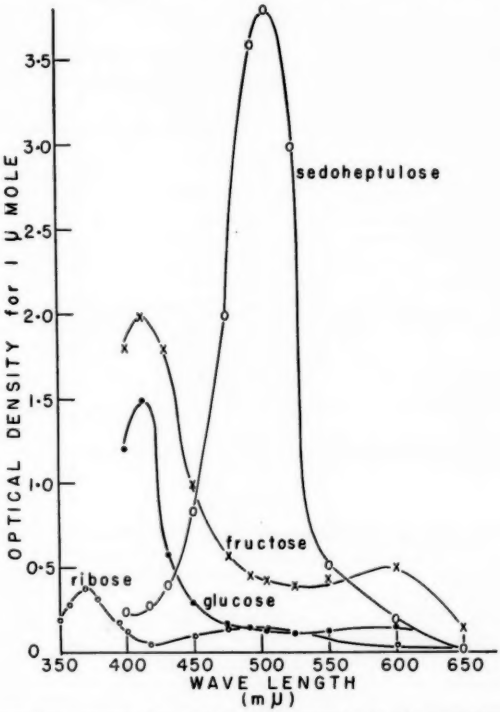

FIG. 2. Absorption curves for various compounds in the cysteine-sulfuric acid method. 1 μmole of each compound was used, except for sedoheptulose, which was 0.5 μmole, and corrections were made to graph as 1 μmole. To 0.5 ml. of a solution of the compound were added 2.5 ml. of the sulfuric acid mixture (20 ml. of H_2O-120 ml. of concentrated H_2SO_4), both components being in an ice bath. The mixture was heated for 3 minutes at 100°, removed, and immersed in an ice bath. Then 0.05 ml. of 3 per cent cysteine was added and the colors were developed for 18 hours.

be noted, but it is felt that many of the values presented may have been overcorrected. This may be responsible in part for the failure to account for all of the carbon in some of the sugar phosphate interconversions.

Results

Early studies indicated that the phosphate esters were not oxidized in the cell-free extracts in a manner exclusively indicative of complete oxida-

tion by way of glycolysis and the tricarboxylic acid cycle. Glucose-6-phosphate (G-6-P) and glucose-1-phosphate (G-1-P) were oxidized more rapidly than fructose-1,6-diphosphate (FDP), as shown in Table II.

TABLE I

Molar Extinction Coefficients for Various Carbohydrates in Colorimetric Measurements Used for Their Determination

Compound	Molar extinction coefficient, sq. cm. per mole		
	Orcinol test, 660 mμ	Cysteine-sulfuric acid test	
		415 mμ	505 mμ
Ribose	2.2×10^4	2.7×10^2	6.4×10^2
Ribose-5-phosphate	2.2×10^4		
Sedoheptulose	3.8×10^3	8.5×10^2	1.1×10^4
Fructose	1.3×10^3	6.1×10^3	1.3×10^3
Glucose	1.1×10^3	4.5×10^3	4×10^2
Dihydroxyacetone	3×10^2	0	0
6-Phosphogluconate	0	0	0

Details of the determinations are given in Figs. 1 and 2.

TABLE II

Oxidation of Hexose Phosphates by Cell-Free Preparation of Pea Aphid

Compound	μatoms of oxygen per μmole of substrate added initially					
	10 min.	20 min.	30 min.	40 min.	50 min.	100 min.
Glucose-6-phosphate	1.3	2.2	2.7	3.6	3.9	4.4
Glucose-1-phosphate	1.0	1.9	2.8	3.5	4.2	4.7
Fructose-1,6-diphosphate	0.8	1.6	2.2	2.4	2.7	3.0

Each Warburg vessel contained 4 μmoles of substrate, 11 mg. of protein, 10 μmoles of nicotinamide, 2 μmoles of Mg^{++}, 1 mg. of Armour coenzyme concentrate, 0.2 mg. of *N*-methylphenazine sulfate, 30 μmoles of phosphate buffer at pH 7.5. Temperature, 28°; total volume, 3.0 ml. Atmosphere, air. The substrate, Armour coenzymes, and phenazine were tipped from a side arm at zero time.

Glucose and fructose were only feebly oxidized, and all attempts to demonstrate hexokinase activity have been unsuccessful. Oxidation of the Krebs cycle acids was more limited than the oxidation of the hexose phosphates, as shown in Fig. 3. In addition, no inhibition of G-6-P oxidation occurred in the presence of fluoride or iodoacetate. Cytochrome *c* was able to serve as an electron carrier, but to a smaller extent than *N*-methylphenazine sulfate. TPN was found to be the active component in the

coenzyme concentrate, although DPN also possessed considerable activity. These relationships are shown in Table III.

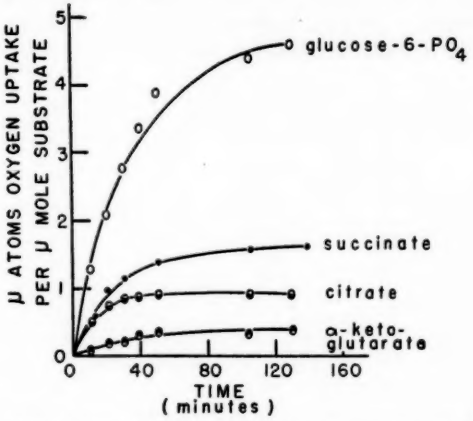

FIG. 3. Oxidation of some tricarboxylic acid cycle components by a cell-free extract from pea aphids. Conditions were as listed in Table II. The results are expressed as microatoms of O_2 uptake per micromole of substrate added to the vessel.

TABLE III

Reduction of Tetrazolium by Cell-Free Preparations of Pea Aphids in Presence of TPN or DPN with Several Substrates

Compound	Tetrazolium reduced per μmole substrate added initially	
	TPN	DPN
	μmole	*μmole*
Glucose-6-phosphate	1.0	0.4
6-Phosphogluconate	0.6	0.33
Ribose-5-phosphate	0.6	0.26
Fructose-6-phosphate	0.7	0.34

The incubation mixtures consisted of 5 μmoles of substrate, 3.8 mg. of protein, 60 μmoles of NaF, 0.13 μmole of TPN (or 0.15 μmole of DPN), 45 μmoles of TTZ, 0.38 μmole of pyocyanine, 40 μmoles of Tris buffer, pH 7.5. Total volume, 1.7 ml.; temperature, 30°; time, 15 minutes. The optical density was read at 505 mμ.

The addition of pyocyanine improved the reduction of tetrazolium. For example, in a 10 minute incubation with G-6-P as substrate, 0.1 μmole of TTZ was reduced per micromole of substrate with this oxidant alone, while 0.18 μmole was reduced per micromole of substrate in the presence of both TTZ and pyocyanine.

G-1-P was rapidly oxidized, probably by conversion to G-6-P. The enzyme phosphoglucomutase has been demonstrated.[2] Aldolase was also detected in the cell-free extract by the method of Bard and Gunsalus (8).

Ribose-5-phosphate (R-5-P), 6-phosphogluconate (6-PGA), and fructose-6-phosphate (F-6-P) are oxidized by the cell-free extract, as shown in

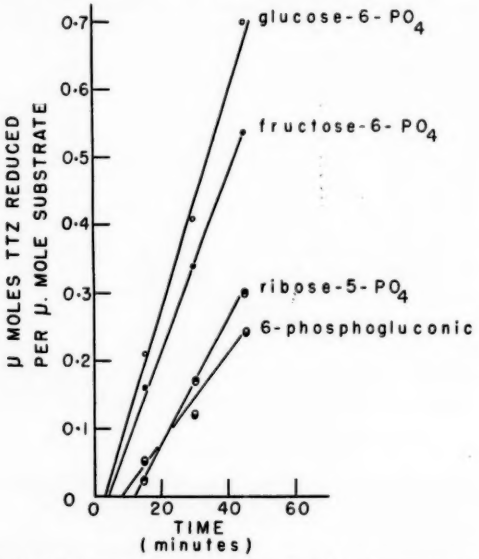

FIG. 4. Oxidation of hexose phosphates by a cell-free preparation of pea aphid. The incubation mixture consisted of 4 μmoles of substrates G-6-P and 6-PGA, 5 μmoles of substrate F-6-P and R-5-P, 45 (1.5 mg.) μmoles of TTZ, 0.07 μmole of TPN; 2.0 mg. of protein, 20 μmoles of Tris buffer, pH 7.5. Total volume, 1.6 ml.; temperature, 30°. The results are expressed as microatoms of O_2 uptake per micromole of substrate added to the vessel.

Fig. 4. F-6-P is oxidized as rapidly as G-6-P, indicating a rapid interconversion of these compounds.

The reactions in the pentose cycle were then studied and their presence indicated as shown in the following experiments.

(1) G-6-P → 6-PGA

Since a pigment present in cell-free extracts of the aphid absorbs strongly at 340 mμ, it was not possible to follow the reduction of TPN. The formation of 6-PGA was demonstrated by cochromatography on paper.

(2) 6 PGA → R-5-P

[2] McGinnis, A. J., Newburgh, R. W., and Cheldelin, V. H., unpublished results.

As shown in Table IV, 6-PGA is converted to pentose, sedoheptulose, and hexose. In addition, the formation of R-5-P and F-6-P was demonstrated by paper chromatography.

(3) R-5-P → sedoheptulose, triose, and hexose

TABLE IV

Formation of Pentose, Sedoheptulose, and Hexose from 6-PGA in Pea Aphid

Compound formed	μmoles formed						
	0 min.	5 min.	15 min.	45 min.	60 min.	90 min.	120 min.
Pentose	0	0	1.2	0.6	1.6	2.5	4.0
Sedoheptulose	0	0	0.15	0.19	0.4	1.1	1.5
Hexose	0	0	0	0	0.3	1.0	1.3

The incubation mixture consisted of 8 μmoles of 6-PGA, 68 mg. of protein, 0.13 μmole of TPN, and 400 μmoles of Tris buffer at pH 7.5. Total volume, 15 ml.; temperature, 30°. Aliquots were removed at the times indicated and mixed with an equal volume of 10 per cent trichloroacetic acid.

TABLE V

Formation of Sedoheptulose, Triose, and Hexose from R-5-P by Pea Aphid

Compound	Amount present, μmoles							
	0 min.	1 min.	5 min.	20 min.	40 min.	60 min.	90 min.	120 min.
Pentose	10	7.7	4.7	3.2	2.3	2.3	2.7	2.4
Sedoheptulose	0	1.3	2.5	2.2	3.0	2.5	2.1	2.0
Triose	0	0.9	1.7	1.3	1.3	1.0	1.0	0.9
Hexose	0	0	0.5	0.8	1.9	1.9	1.7	2.0
Total*	10	10	9.8	8.0	8.1	8.1	8.0	7.9

The incubation mixtures consisted of 10 μmoles of R-5-P, 70 mg. of protein, 400 μmoles of Tris buffer, pH 7.5. Total volume, 10 ml.; temperature, 30°; aliquots removed as in Table IV.

* Micromoles of carbon divided by 5.

Table V depicts the formation of triose, SH, and hexose with the disappearance of pentose, when R-5-P is used as substrate. The very rapid appearance of SH and triose and the more gradual accumulation of hexose, are to be expected from pentose cycle activity, and recall the similar results obtained with *Acetobacter* (6).

(4) F-6-P → G-6-P

This reaction was followed in the reverse manner by measuring the

formation of fructose from G-6-P in the absence of TPN. 5 μmoles of fructose were formed in 30 minutes from 10 μmoles of G-6-P. In addition, cochromatography indicated the formation of G-6-P from F-6-P.

(5) Cofactors for oxidation of FDP

It was difficult with the preparations described to demonstrate an effect of Mg^{++} in the systems studied. This was made possible, at least for the oxidation of FDP, by dialyzing the cell-free extract for 3 hours at 0° against Dowex 50 (hydrogen form) in the outer cell. This treatment apparently also removed FMN or other flavins, since increased activity was obtained when small amounts of FMN were added to the dialyzed

TABLE VI

Effect of Mg^{++} and FMN on Oxidation of FDP and G-6-P in Pea Aphid

	μmole of TTZ reduced per μmole substrate added initially	
	FDP	G-6-P
Complete system	0.22	0.42
No Mg^{++}	0.017	0.44
" FMN	0.18	0.42
" Mg^{++}, FMN	0.005	0.44

The incubation mixture consisted of 3.2 mg. of protein, 0.13 μmole of TPN, 45 μmoles of tetrazolium, 0.38 μmole of pyocyanine, 0.05 μmole of FMN, 2 μmoles of Mg as indicated, 4 μmoles of G-6-P, 20 μmoles of Tris buffer, pH 7.5. Total volume, 1.6 ml.; temperature, 30°; time, 15 minutes.

preparation. The results are shown in Table VI. Neither Mg nor FMN had any effect on the oxidation of glucose-6-phosphate.

DISCUSSION

Evidence has been accumulating that the pentose cycle is operative in a variety of organisms (4–7). The studies in this and the preceding papers indicate that this cyclic mechanism, which can account for the oxidation of hexoses to carbon dioxide and water, occurs in even more unrelated species. This broad distribution poses the question of the general importance of this pathway of carbohydrate oxidation. If, as suggested by Krebs *et al.* for yeast (9), the Krebs cycle should serve mainly to synthesize amino acids or other essential metabolites, then the pentose cycle might function in some organisms as a major energy source. This would seem especially worthy of consideration in *Acetobacter*, where Krebs cycle activity is slight (6, 10). Presumably, an apparatus for oxidative phos-

phorylation is present in each of the species containing the pentose cycle, since pyridine nucleotide-linked oxidations occur; phosphorylations coupled to pyridine nucleotide oxidations have been amply demonstrated (11). The use of pentose cycle carbon for organic syntheses is also possible, as in nucleotide formation or in carboxylation of pentose (12, 13), although on the basis of present knowledge the Krebs cycle might be expected to provide a greater variety of metabolic intermediates. It would appear that a continued evaluation of the relation of the pentose cycle to carbohydrate oxidation, the energetics of the cell, and to mitochondrial activity (in which the Krebs cycle activity resides) is necessary. Studies of these relationships are in progress.

The authors gratefully acknowledge the advice and counsel of Dr. P. O. Ritcher of the Department of Entomology, Oregon State College, who suggested the organism to be used, and directed the obtaining of the pea aphid in quantities sufficient to use for enzyme studies.

SUMMARY

The oxidation of carbohydrates by way of the pentose cycle has been demonstrated to occur in the pea aphid. The implications of this finding and the presence of this cyclic mechanism in other organisms have been discussed.

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A STUDY OF THE MECHANISM OF SERINE BIOSYNTHESIS*

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(Received for publication, September 21, 1954)

Elwyn *et al.* (1, 2) have demonstrated that the C_1 unit formed from serine is of the oxidation level of formaldehyde rather than formate. They compared the dilution of the C^{14} and deuterium of 2,3-deuterio-3-C^{14},N^{15}-L-serine that occurred during the conversion of the serine β-carbon to methyl groups in rats. The results indicated that this carbon did not pass through an intermediate, such as formate, which required the loss of 1 of the hydrogen atoms. In consideration of the results of Elwyn *et al.* and the general implication of the citrovorum factor, 5-formyl-5,6,7,8-tetrahydrofolic acid, in the metabolism of C_1 compounds, Welch and Nichol (3) proposed that the C_1 unit formed from serine was a reduced form of citrovorum factor, 5-hydroxymethyl-5,6,7,8-tetrahydrofolic acid, a hypothetical condensation product of formaldehyde and tetrahydrofolic acid.

The formulation of the biosynthesis of serine as a process involving a condensation between the α-carbon of glycine, activated by the Schiff base formation with pyridoxal phosphate (4), and 5-hydroxymethyl-5,6,7,8-tetrahydrofolic acid is reminiscent of the Mannich reaction (5). Serine would be produced by hydrolytic cleavage of the resulting product. The conversion of serine to glycine could occur by the reverse process.

The participation of THFA[1] as a cofactor in the biosynthesis and cleavage of serine has been studied by adding it to pigeon liver extracts incubated anaerobically with glycine-1-C^{14} and L-serine. The assumption underlying this procedure was that the formation and breakdown of serine were catalyzed by a single enzyme. In this case, the β-carbon atom of the L-serine would provide the C_1 unit for the conversion of glycine-1-C^{14}

* The results of preliminary experiments in this investigation have been published (*J. Am. Chem. Soc.*, **76**, 1456 (1954)).

This investigation has been supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[1] The following abbreviations are employed in this paper: THFA = 5,6,7,8-tetrahydrofolic acid; DHFA = 7,8-dihydrofolic acid; FA = folic acid; THFA-CH_2OH = 5-hydroxymethyl-5,6,7,8-tetrahydrofolic acid; THFA-CHO = citrovorum factor; ATP = adenosinetriphosphate; G-6-P = glucose-6-phosphate; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide.

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to serine-1-C^{14}. The sole addition of tetrahydrofolic acid produced a

$$CH_2OHCHNH_2COOH \rightleftarrows C_1 + CH_2NH_2COOH$$

$$C_1 + CH_2NH_2C^*OOH \rightleftarrows CH_2OHCHNH_2C^*OOH$$

pronounced stimulation of the incorporation of glycine-1-C^{14} into serine even when the extract had been treated previously with Dowex 1 chloride and dialyzed. The incorporation of formaldehyde and formate into serine has also been studied, and it has been possible to reactivate treated[2] extracts by the addition of THFA and, in the case of formate, of ATP and a reducing system.

EXPERIMENTAL

Materials and Methods

5,6,7,8-Tetrahydrofolic acid was prepared by the method of Broquist *et al.* (6), 7,8-dihydrofolic acid by the procedure of O'Dell *et al.* (7), formate-C^{14} according to Melville *et al.* (8), and formaldehyde-C^{14} by the procedure of Siegel and Lafaye (9).

Male pigeons were obtained from the Palmetto Pigeon Plant.

Phosphate buffer extracts of pigeon liver were prepared by the procedure of Berg (10), lyophilized, and stored *in vacuo* at −10°. Extracts were treated with Dowex 1 chloride (50 to 100 mesh, 4 per cent cross-linked) by the following procedure: 880 mg. of the lyophilized powder were dissolved in 8 ml. of ice-cold distilled water and stirred with 3 gm. of Dowex resin (prepared by the method of Chantrenne and Lipmann (11) and dried in air) in an ice bath for 10 minutes. After removal of the resin by centrifugation at 0°, the extract was diluted with an equal volume of cold 0.2 M potassium phosphate buffer, pH 7.5, and dialyzed in Visking NoJax casing (8/32 inches) suspended in flowing 0.1 M potassium phosphate buffer, pH 7.5, at 2°. Extracts treated with Dowex 1 chloride and dialyzed were stable toward lyophilization.

Incubation was carried out in Thunberg tubes at 34°. The radioactive substrate was placed in the side arm and all other components of the incubation mixture were added to the main compartment which was immersed in an ice bath. The gas phase was hydrogen at atmospheric pressure. At the end of the incubation 1 ml. of 20 per cent trichloroacetic acid was added and the mixture was centrifuged. The value for the "total activity fixed" (Table V) was determined by adding 0.1 ml. of the supernatant solution to 2 ml. of 1 N HCl in a Pyrex ashing dish and evaporating to dryness under an infra-red lamp. The activity of the residue was determined with an end window Geiger-Müller counter.[3] Serine and

[2] Treated with Dowex 1 chloride and dialyzed.

[3] The self-absorption of these samples was determined to be negligible.

glycine were isolated from aliquots of the protein-free incubation mixture by chromatography on Dowex 50 (100 to 200 mesh, 12 per cent cross-linked) with 1 N HCl as the eluent. When radioactive glycine as well as serine was present, 50 × 1 cm. columns were used; otherwise 25 × 1 cm. columns were employed. The activity of the serine and glycine was determined by evaporating aliquots of the respective fractions on Pyrex ashing dishes and measuring radioactivity as in the determination of total "fixed" activity.[3] This procedure did not cause loss of activity of the samples.

The identification of the activity of the serine fraction of the Dowex chromatogram with serine was further established by ascending paper chromatography with Whatman No. 4 filter paper and 80 per cent pyridine-water as the solvent. All of the radioactivity on the paper was located in a single spot possessing the R_F of L-serine.

Serine was determined by a modification of the procedure of Frisell *et al.* (12) in which formaldehyde resulting from the periodate oxidation of serine was determined with Schiff's reagent rather than with chromotropic acid. In our hands this modification permitted greater precision.

The activity of the β-carbon of serine was determined by dilution of the formaldehyde formed from serine by periodate oxidation with carrier formaldehyde and conversion to the dimedon derivative. An aliquot of an alcohol solution of the formaldehydemethone was evaporated on a Pyrex ashing dish and counted.[3] Protein was determined by the biuret method (13).

Results

Pigeon liver extracts possess varying abilities to interconvert glycine and serine. This ability is slowly lost on storage of the lyophilized powder. The extract used in the experiment shown in Table I had been stored for several months and had retained very little activity. The sole addition of THFA produced a marked increase in the radioactivity of the serine. It has not been possible to demonstrate any other requirement of this reaction. When the extracts were treated with Dowex 1 chloride and subjected to prolonged dialysis, they were restored to the activity of the original preparation supplemented with THFA by the sole addition of THFA (Table I). The further addition of ATP, DL-homocysteine, or DPN (Table II) produced no additional activation. The incorporation of formate-C^{14} into serine (10) and the interconversion of glycine and serine[4] in untreated pigeon liver extracts are stimulated by DL-homocysteine. However, homocysteine does not increase the incorporation of glycine into serine in treated[2] extracts with or without THFA supplementation.

[4] Unpublished experiments of R. L. Kisliuk and W. Sakami.

Folic acid could not replace tetrahydrofolic acid in stimulating the incorporation of glycine-C^{14} into serine and was inhibitory when added together with THFA.

TABLE I

Effect of THFA on Incorporation of Glycine-1-C^{14} into Serine

Preparation	Addition	Total activity of serine
		c.p.m.
1. Untreated extract	None	200
2. " "	THFA	7600
3. Treated extract	None	300
4. " "	THFA	7600
5. " "	FA	0
6. " "	THFA + FA	5200

1 ml. of untreated or Dowex-treated, dialyzed (15 hours) extract (30 mg. of protein), 3.5 μmoles of glycine-1-C^{14} (16,600 c.p.m. total), 8 μmoles of L-serine, 3.5 μmoles of THFA, 3.0 μmoles of FA, 100 μmoles of potassium phosphate buffer, pH 7.5. Total volume 2 ml. Incubation 1 hour.

TABLE II

Effect of Various Additions on Incorporation of Glycine-1-C^{14} into Serine

Additions	Activity of serine
	c.p.m.
1. None	1180
2. DL-Homocysteine	1200
3. THFA	2770
4. " + ATP	2730
5. " + DL-homocysteine	2760
6. " + DPN	2790
7. " (45 min. incubation)	4310

0.5 ml. of Dowex-treated, dialyzed (22 hours) extract (14 mg. of protein), 10 μmoles of glycine-1-C^{14} (11,000 c.p.m.), 10 μmoles of L-serine, 5.0 μmoles of DL-homocysteine, 3.5 μmoles of THFA, 10 μmoles of ATP, 1.0 μmole of DPN, 100 μmoles of potassium phosphate buffer, pH 7.5. Total volume 2 ml. Incubation 11 minutes.

Results similar to these have been briefly reported by Blakley (14).

The incorporation of formaldehyde and formate into serine was studied by incubating pigeon liver extracts with formaldehyde-C^{14} or formate-C^{14} and glycine and determining the amount and the total activity of the serine formed. The addition of THFA to inactivated extracts strongly stimulated the incorporation of formaldehyde-C^{14} into serine (Tables III and IV). The specific activity of the serine and of the serine β-carbon was

determined (Table IV) and found to be equal to that of the formaldehyde used in the incubation within the experimental error. This demonstrated

TABLE III

Incorporation of Formaldehyde-C^{14} into Serine in Pigeon Liver Extract

Preparation	Additions	Activity in serine
		c.p.m.
Treated	None	180
"	THFA	112,000
"	" + ATP	124,000
"	" + DL-homocysteine	54,000
"	DHFA	7,500
Untreated	None	1,350
"	THFA	96,000
"	" + ATP	90,000
"	" + DL-homocysteine	51,000
"	DHFA	47,000

1 ml. of extract untreated (37 mg. of protein) or treated twice with Dowex 1 chloride and dialyzed (24 hours) (25 mg. of protein), 6.5 μmoles of HCHO-C^{14} (280,000 c.p.m.), 10 μmoles of glycine, 10 μmoles of ATP, 5 μmoles of DL-homocysteine, 3.5 μmoles of THFA, 3.5 μmoles of DHFA. Total volume 2 ml. Incubation 13 minutes.

TABLE IV

Effect of Folic Acid Derivatives on Formaldehyde Utilization

Additions	None	FA	DHFA	THFA
	Activity in serine			
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
1. None	110	140	3,650	67,500*
2. ATP		110	3,850	61,000
3. " DPN, $MnSO_4$, G-6-P		580	54,000	61,000

1 ml. of Dowex 1-treated, dialyzed (24 hours) extract (40 mg. of protein), 6.5 μmoles of formaldehyde-C^{14} (280,000 c.p.m.), 10 μmoles of glycine, 3.5 μmoles of FA, 3.5 μmoles of DHFA, 3.5 μmoles of THFA, 10 μmoles of ATP, 1.0 μmole of DPN, 1.0 μmole of $MnSO_4$, 10 μmoles of glucose-6-phosphate, 100 μmoles of potassium phosphate buffer, pH 7.5. Preincubation 7 minutes before tipping in formaldehyde. Total volume 2 ml. Incubation 5 minutes.

* The specific activities of the serine, the serine β-carbon isolated, and the formaldehyde added to the incubation mixture were 45,000, 41,300, and 43,000 c.p.m. per μmole, respectively.

that the incorporation of formaldehyde activity into serine occurred by a net synthesis. As in the case of the interconversion reaction of serine and glycine, it has not been possible to detect any other cofactor requirement of

this reaction. The activity of the treated[2] extract stimulated by THFA was approximately equal to that of the untreated pigeon liver preparation similarly supplemented (Table IV). Moreover, additional supplementation with ATP or with a mixture of ATP, DPN, Mn^{++}, and glucose-6-phosphate produced no further effect in either preparation. This mixture was added with the assumption that it would reconstitute the glycolytic system of pigeon liver extract, thereby increasing the supply of ATP. It is also provided reduced DPN. Homocysteine decreased the activity

TABLE V

Effect of Tetrahydrofolic Acid and Other Substances on Incorporation of Formate-C^{14} into Serine β-Carbon

Experiment No.		Activity of serine	Total activity fixed
		c.p.m.	*c.p.m.*
1	None	20	
	THFA	220	
	" + ATP + DPN + $MnSO_4$ + G-6-P	79,000*	
	ATP + DPN + $MnSO_4$ + G-6-P	180	
2	THFA + ATP + DPN + $MnSO_4$ + G-6-P	98,000	95,000
	" less ATP	6,500	6,300
	" " DPN	27,000	45,000
	" " G-6-P	1,600	82,000
	" " $MnSO_4$	28,000	28,500
	Untreated extract + THFA + ATP + DPN + $MnSO_4$ + G-6-P	100,000	112,000

1 ml. of Dowex 1-treated, dialyzed (24 hours) extract (Experiment 1, 45 mg. of protein, Experiment 2, 41 mg. of protein) or untreated extract (45 mg. of protein), 5 μmoles of formate-C^{14} (270,000 c.p.m.), 10 μmoles of glycine, 3.5 μmoles of THFA, 10 μmoles of ATP, 1.0 μmole of DPN, 2.0 μmoles of $MnSO_4$, 20 μmoles of G-6-P. Total volume 2 ml. Incubation 30 minutes.

* The specific activities of the serine, the serine β-carbon, and the formate added to the medium were 59,000, 51,000, and 54,000 c.p.m. per μmole, respectively.

incorporated into serine as would be expected according to its ability to bind formaldehyde (10). DHFA was much more strongly stimulatory in the untreated than in the treated extract (Table IV). Although the addition of DHFA produced very low serine activity when added to the treated[2] extract, further supplementation of the extract with ATP, DPN, Mn^{++}, and glucose-6-phosphate raised the activity of the serine almost to that produced by the addition of THFA. FA was slightly active under these conditions.

Similar results have been briefly reported by Blakley (14).

The addition of THFA alone to treated[2] pigeon liver extracts did not produce a significant increase in its ability to incorporate formate-C^{14} into

serine (Table V). This was not unexpected, since the conversion of formate to the serine β-carbon involves a reductive step. The further addition of ATP, DPN, $MnSO_4$, and glucose-6-phosphate produced a marked incorporation of formate carbon into serine (Table V). The specific activity of the serine and of the serine β-carbon was determined (Table V) and found to be approximately equal to that of the formate, indicating that the introduction of formate-C^{14} into serine occurred by a net synthesis. The activity of the serine was similar to that produced by untreated enzyme supplemented in the same manner (Table V).

In agreement with the report of Berg (10), homocysteine stimulated the incorporation of formate-C^{14} into serine in the untreated extract. However, it did not significantly activate the treated[2] preparation. The effect of single omissions from the additions of ATP, DPN, $MnSO_4$, and glucose-6-phosphate on the activity of the serine has been determined. In the absence of any one of these substances the radioactivity of the serine was diminished. The absence of DPN or glucose-6-phosphate resulted in an accumulation of formate-C^{14} in some compound that was not serine. The nature of this substance is being investigated. These results suggest that reduced DPN is not required in the initial phase of formate utilization.

DISCUSSION

The finding that the conversion of glycine-C^{14} to serine-C^{14} in the presence of L-serine is strongly stimulated by the sole addition of THFA[5, 6] supports the hypothesis that the formation of serine from glycine and the breakdown of serine are catalyzed by a single enzyme for which the name serine hydroxymethylase is proposed since it catalyzes the removal and addition of a hydroxymethyl group. It is uncertain whether THFA itself, or a derivative, is the physiological cofactor of this reaction. Folic acid and citrovorum factor occur in animal tissues largely in bound form (15–19) and it is possible that the cofactor of serine hydroxymethylase is a similar derivative of THFA.

The scheme relating formate, formaldehyde, and serine shown in Fig. 1 is tentatively proposed to account for the results obtained in the present investigation. This scheme is similar to the one proposed by Berg (10) except that homocysteine has been replaced by THFA.[5] According to the present hypothesis, the C_1 unit directly involved in serine biosynthesis is THFA-CH_2OH.[5] Formaldehyde is utilized for the formation of the serine β-carbon by enzymatic condensation with THFA[5] producing THFA-

[5] Or a derivative.

[6] Serine hydroxymethylase has been purified by precipitation with ammonium sulfate (between 25 and 40 per cent saturated at 2°). This preparation possessed 4 times the specific activity of the original and was activated by the sole addition of THFA.

CH_2OH.[5] Formate utilization is assumed to occur by a pathway in which THFA[5] and formate are combined in the presence of ATP and Mn^{++} to form THFA-CHO.[5] THFA-CHO[5] is then reduced to THFA-CH_2OH[5] by a DPN enzyme system. It is also possible that TPN rather than DPN is involved in this reduction. DPN is converted to TPN in liver in the presence of ATP (20).

The enzymatic nature of the formation of THFA-CH_2OH[5] from THFA[5] and formaldehyde has been indicated by studies of deoxycholic acid extracts of rat liver particles.[7] When supplemented with THFA, this preparation catalyzes the rapid interconversion of serine and glycine but does

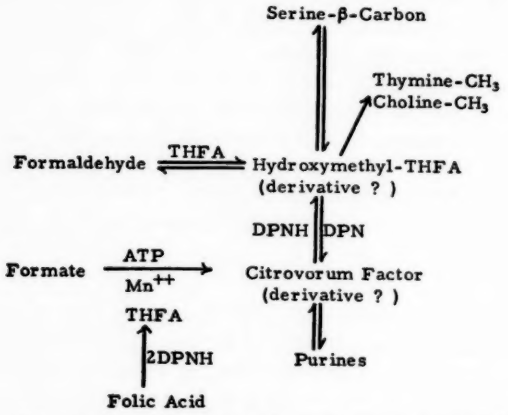

FIG. 1. Interrelationships between 1-carbon compounds

not utilize formaldehyde as would be expected if the formation of THFA-CH_2OH were solely a spontaneous process.

The effect of single omissions from the mixture of ATP, DPN, Mn^{++}, and glucose-6-phosphate is consistent with this scheme. When ATP or Mn^{++} was omitted, the amount of formate-C^{14} incorporated into non-acid-volatile substances was considerably reduced and all of this activity was accounted for as serine. These findings are consistent with a rôle of ATP and Mn^{++} in the initial process of formate utilization. When DPN or glucose-6-phosphate was omitted, considerable formate was converted to a substance that was not serine but which may have been citrovorum factor. The formation of citrovorum factor from folic acid in liver has been observed by Nichol (21). These results are consistent with the hypothesis that the initial step in formate utilization does not involve reduction and that formate is not converted to serine via formaldehyde.

[7] Unpublished experiments of S. Deodhar and W. Sakami.

The scheme shown in Fig. 1 is consistent with the literature on the relationships of compounds possessing a single carbon atom. It is also consistent with the indication that formate and formaldehyde do not undergo obligatory interconversion in their incorporation into carbons 2 and 8 of purine (22) but are metabolized via a common intermediate. Furthermore, the scheme agrees with tracer studies showing that the carbons of β-deuterioserine-C^{14} and deuterioformate-C^{14} are incorporated into choline (23, 1, 24) and thymine (1, 24) methyl groups with little or no loss of deuterium. In the case of the formate utilization, the interconversion of citrovorum factor and THFA-CH_2OH would not be expected to produce loss of carbon-bound deuterium. Hydrogen would be expected to add to the formyl group in the reduction of the citrovorum factor, as in most biological reactions, with a single spatial distribution and would be the same hydrogen atom removed in the reversal of this process. In the case of serine, the results are consistent with the existence of a THFA-CH_2OH intermediate which can undergo rapid reversible conversion to citrovorum factor though not with obligatory conversion to the latter compound. Ratios for C^{14}:D dilution of 0.97 to 0.89 (1) and of 0.75 and 0.78 (1, 24) for the conversion of serine β-$C^{14}D_2OH$ to choline methyl groups and to thymine methyl groups, respectively, have been reported. Conversion of THFA-CH_2OH to THFA-CHO and back to THFA-CH_2OH would result in a ratio of 0.50. Repetition of this process would not be expected to decrease the ratio further, since the hydrogen with a single spatial distribution would be removed and added in each oxidation and reduction. Since the THFA-$C^{14}DO$ may be highly diluted by a pool of unlabeled citrovorum factor, the THFA-$C^{14}DHOH$ produced from it may contribute relatively little C^{14} or deuterium to methyl groups, compared with THFA-$C^{14}D_2OH$ directly formed from serine. Under these conditions the THFA-CH_2OH and THFA-CHO could undergo extensive interconversion with little effect on the ratio of C^{14}:D dilution of the thymine or choline methyl groups.

The participation of citrovorum factor in purine synthesis and breakdown in pigeon liver has been indicated by recent reports. Buchanan and Schulman (25) observed a stimulatory effect of leucovorin on the incorporation of radioactive formate into inosinic acid in a pigeon liver enzyme system. Greenberg (26) later observed the formation of a substance from leucovorin and ATP that converted 5-amino-4-imidazolecarboxamide to inosinic acid. Flaks and Buchanan (27) have reported the stimulation of the reverse process by the natural citrovorum factor, glycine, and copper. The conversion of both the serine β-carbon and formate to carbons 2 and 8 of purine involves some process in which a labilization of the carbon-bound hydrogen occurs (24).

The rôle of homocysteine in serine formation is uncertain. Berg tentatively postulated the existence of *S*-formyl- and *S*-hydroxymethylhomocysteine intermediates of formate metabolism to explain the specific stimulatory effect of homocysteine on the incorporation of formate-C^{14} into serine β-carbon in untreated pigeon liver extracts (10). Deodhar subsequently found that homocysteine stimulates and cysteine inhibits the incorporation of formate-C^{14} into serine β-carbon in untreated chicken liver extracts.[7] Homocysteine does not, however, stimulate the interconversion of serine or glycine or the utilization of formaldehyde and formate in Dowex-treated, dialyzed extracts supplemented with THFA. Studies of Doctor and Couch (18) suggest that homocysteine may be involved in the reduction of folic acid to tetrahydrofolic acid. Homocysteine was reported to increase the conversion of folic acid to citrovorum factor; glutathione, ascorbic acid, and cysteine were without effect.

SUMMARY

The incorporation of glycine and formaldehyde-C^{14} into serine in Dowex 1-treated and dialyzed pigeon liver extracts has been reactivated by the sole addition of 5,6,7,8-tetrahydrofolic acid. Formate-C^{14} incorporation has been stimulated by the addition of tetrahydrofolic acid, together with ATP, DPN, Mn^{++}, and glucose-6-phosphate. The introduction of formaldehyde and formate-C^{14} into serine occurred by net synthesis of the amino acid. The implications of these findings have been discussed.

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DETERMINATION OF NUCLEIC ACIDS IN ANIMAL TISSUES

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As reported in a previous paper (1), a new microchemical method for the determination of deoxyribonucleic acid (DNA) has been devised which permits the evaluation of DNA in amounts as small as 2.5 γ per ml. The method is particularly useful with very small quantities of tissue. For this reason a study was undertaken to learn the best conditions for the application of the method to the determination of DNA in animal tissues. The reaction of DNA with indole was compared with the diphenylamine reaction according to Dische (2). At the same time, a modification of the Barrenscheen and Peham (3) method for ribose determination with orcinol was worked out in order to determine pentose nucleic acid (PNA) in the same organ extracts. Total nucleic acid P of tissues was calculated from the PNA and DNA contents, found by means of color reactions. These values were compared to those obtained by a direct chemical method. Readings were also taken at 260 and 268 mμ and their values compared with those calculated from the results of the color reactions.

EXPERIMENTAL

DNA Reaction with Indole—Since DNA was to be determined in tissue extracts, it was necessary to test the effect of the usual nucleic acid extraction media (trichloroacetic acid, $HClO_4$, NaOH) on the indole reaction.

The standard curve was made with a sample of DNA from calf thymus, prepared according to the Hammarsten method and having the following characteristics: N 13.4, P 8.0, adenine 10.0, guanine 7.4, cytosine 4.7, thymine 8.4 per cent, N:P 1.68 (from Dr. L. Cavalieri).

The DNA reaction with indole[1] was performed under several different conditions. Previous boiling of the DNA solution in 0.1 N NaOH for half an hour did not affect the results. This was also true when the solution in N KOH was incubated for 24 hours at 37°. The small amount of alkali introduced into the reaction did not cause any variation in the results; however, when used for tissue extraction, alkaline treatment presented

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[1] The indole was purified by distillation under reduced pressure.

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several disadvantages. With organs rich in lipides or connective tissue, the extracts are never completely clear; furthermore, consistently lower values were obtained for PNA determinations than with the perchloric acid procedure.

Trichloroacetic acid, in the amounts usually employed for deproteinization, reacts with indole, giving a pink, cloudy color that is extracted completely by chloroform. At the same time it inhibits the reaction between indole and DNA.

Perchloric acid tested in a final concentration of 3.3 per cent, which is about 3 times that usually employed in the test, did not enhance or decrease the intensity of the color formed during the reaction.

Pentose Reaction with Orcinol—In 1941 Barrenscheen and Peham (3) reported that the Bial reaction can be improved by substituting cupric chloride for ferric chloride. They emphasized the fact that the boiling time should be exactly 10 minutes, because longer boiling increases the intensity of the color and causes a precipitation of the colored compound even at relatively low concentrations; they pointed out also the different slopes of the absorption curves given by arabinose, xylose, adenosine, and adenosinetriphosphoric acid under the same experimental conditions. As Albaum and Umbreit (4) have demonstrated, the different slopes of these curves are due to the different rates of furfural formation from the compounds under consideration. For adenosinetriphosphoric acid, the furfural yield is completed in 10 minutes of hydrolysis; for the other compounds more time is required. After 40 minutes of boiling the pentose and pentose purine nucleosides and nucleotides give all the furfural accounted for by their ribose content; no furfural is formed from pyrimidine nucleotides. Also with PNA the color intensity increases until 40 minutes hydrolysis and then remains almost constant.

At the low concentrations used (2 to 20 γ of ribose per ml.), and after prolonged hydrolysis, formation of precipitates has never been observed. Precipitates form at higher concentrations and eventually, when trichloroacetic acid is present, in rather large amounts. After 10 minutes of hydrolysis, the color formed is blue; it then turns green. After 40 minutes of hydrolysis the color intensity remains constant or decreases only slightly. The color is easily extracted without any change by either amyl or isoamyl alcohol. By this means the color can be concentrated and the sensitivity of the reaction thus increased. Also when the color is precipitated, it dissolves completely in the alcohol.

The reaction is performed according to the following procedure.

Reagents—0.004 M $CuCl_2 \cdot H_2O$ in concentrated HCl (density 1.19); concentrated HCl, c.p. (density 1.19); orcinol freshly recrystallized (m.p. 106°). Solution to be tested, at concentrations of ribose ranging from 2 to 20 γ

per ml. Isoamyl alcohol (b.p. 128.5°), freshly redistilled and filtered after shaking with Darco G-60.

200 mg. of orcinol are dissolved in concentrated HCl; 10 ml. of the $CuCl_2 \cdot H_2O$ solution are added, and the volume is made up to 100 ml. with concentrated HCl. To 5 ml. of the solution to be tested, 5 ml. of the reagent are added. The test-tubes are shaken thoroughly and immersed in a

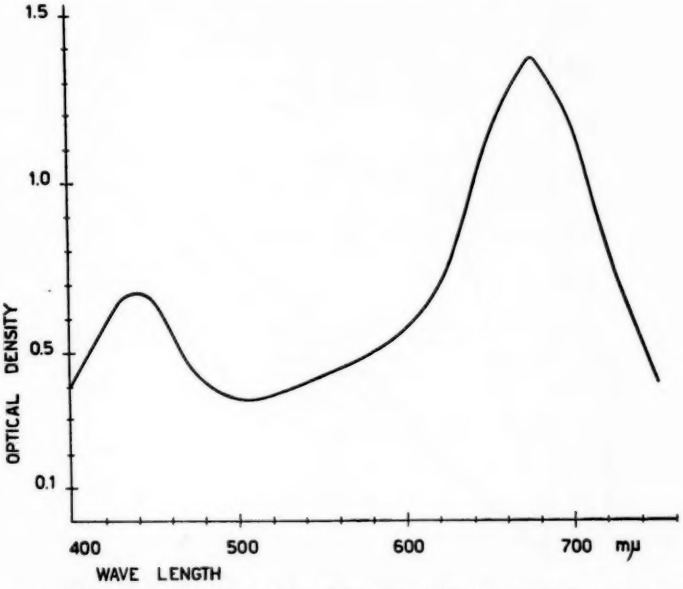

FIG. 1. Absorption spectrum of the color formed by the reaction of ribose and PNA with orcinol.

boiling water bath for 40 minutes; then they are cooled under running water.

The color is extracted with 5 ml. of isoamyl alcohol, and, after centrifugation, is read on a Beckman spectrophotometer at 675 mμ against a blank treated in the same manner (see Fig. 1).

Lambert-Beer's law is followed at concentrations ranging between 2 and 15 γ of ribose per ml. The results are constant and reproducible, and the color is stable. The optical density for 1 γ of ribose per ml. is 0.0715 (Fig. 2).

The results given by PNA were especially studied.

The PNA used presented the following characteristics: N 14.5, P 8.3 per cent, N:P 1.74; content of purine and pyrimidine bases in millimoles per

gm., guanine 0.86, adenine 0.64, cytidine 0.56, uridine 0.50, ribose 2.56 gm. (38.4 per cent). The purine-bound ribose corresponds to 57.6 per cent of the total ribose. From several tests at different concentrations of PNA, the ribose hydrolyzed was found to be 59.87 per cent of the total,

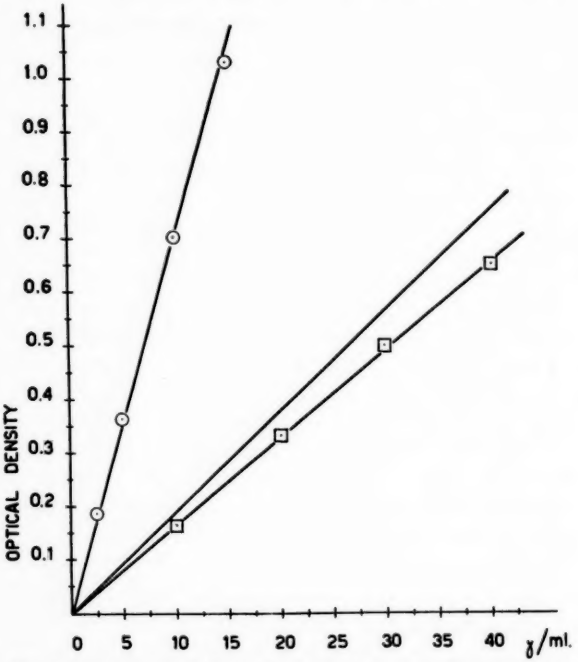

FIG. 2. Absorption curves of the orcinol reaction. ⊙, ribose; ⊡, PNA (P content 8.3 per cent; purine-bound ribose 57.6 per cent). The straight line indicates the theoretical absorption for a PNA containing 9.5 per cent P.

with a deviation in the different tests of ±1.94. These differences are within the limits of error of the method and therefore are not significant.

Practically, only the purine-bound ribose was hydrolyzed. On the basis of the data reported above, the average ratio between the optical density for 1 γ of ribose and 1 γ of PNA is 3.76.

This factor can be used to determine the PNA content of an unknown sample on the basis of the curve for ribose. When the PNA was dissolved in 0.1 N NaOH solution and boiled for half an hour before performing the reaction, the data obtained were the same as with a neutral unboiled solution. DNA solutions at a concentration of 2 mg. per ml., when submitted

to the orcinol test, gave a color corresponding to 4.5 γ of ribose per ml.; that is, to 17.0 γ of PNA. This represents an interference of 0.85 per cent.

Determinations by Ultraviolet Absorption—These were carried out both at 260 and 268 mμ. In fact, as Ogur and Rosen (5) have pointed out, the absorption maximum for PNA is at 260 mμ, for DNA at 268 mμ, when the readings are performed in perchloric acid solution. The optical density for PNA was 0.316 at 260 mμ and 0.298 at 268 mμ per γ of P (Table I). The optical density for PNA appeared to be the same when the readings were made in 0.1 N NaOH as those in 10 per cent perchloric acid solution. On the contrary, the DNA absorption decreased in 0.1 N NaOH solution compared with that in 10 per cent perchloric acid.

TABLE I

Optical Densities of PNA and DNA with Different Reactions

The optical densities per microgram of nucleic acid have been calculated for DNA and PNA corresponding to the classical tetranucleotide formula; therefore P = 9.89 and 9.5 per cent, respectively.

Optical density	DNA				PNA		
	Indole reaction	Diphenylamine reaction	Ultraviolet* at 260 mμ	Ultraviolet* at 268 mμ	Orcinol reaction	Ultraviolet* at 260 mμ	Ultraviolet* at 268 mμ
Per γ P	0.228	0.0265	0.274	0.286	0.200	0.316	0.298
" " nucleic acid	0.0225	0.00263	0.0270	0.0284	0.019	0.0300	0.0283

* In 10 per cent perchloric acid solution.

Phosphorus Determination—The P was determined, after incineration with 0.2 ml. of perchloric acid at 250°, by the amidol (2,4-diaminophenol dihydrochloride) reagent (6). The weights of the different organs ranged usually between 0.5 and 2.0 mg. (dry weight) according to their nucleic acid content. Samples of 0.4 to 1.5 ml. of the tissue extracts were made up to 2 ml. with 10 per cent perchloric acid and then incinerated.

Preparation of Organ Extracts—Extraction of organs is based on the technique described by Ogur and Rosen (5). Immediately after the animal was sacrificed, the organs were minced, weighed, and extracted twice with absolute alcohol and twice with ether. After drying, the residue was extracted with cold 2 per cent perchloric acid in the refrigerator for 20 minutes and then centrifuged in the cold. The supernatant fluid was discarded. The procedure was repeated. The residue was then extracted with 10 per cent perchloric acid at 70° for 20 minutes, centrifuged, the supernatant fluid saved, and the residue extracted again. The combined supernatant solutions were diluted to provide an estimated concentration

ranging between 10 and 15 γ of DNA per ml. and about 25 to 35 γ of PNA per ml.

An alternative technique was often used. The organs were left in absolute alcohol for a few hours, and then the alcohol was changed. They could be stored in alcohol, then in alcohol-ether and ether, for a few weeks, and subsequently dried completely in a desiccator without any noticeable variation in DNA and PNA content. After determination of the dry weight, the perchloric acid extractions were performed as described above.

The amount of tissues used and the final dilution varied according to the estimated difference in nucleic acid content. Usually the extraction was performed with 1 ml. of 10 per cent perchloric acid per 3 to 4 mg. for liver and kidney, 1.5 to 2 mg. for spleen, lung, and pancreas, and 0.5 mg. for thymus. The perchloric acid extracts were further diluted 6 to 12 times with distilled water for the indole reaction. The final perchloric acid content was thus lower than that used for testing its eventual influence on the reaction.

TABLE II

Recovery of DNA from Spleen Extract by Indole Reaction

DNA of spleen extracts, 31.7 γ per ml.

DNA added	Total DNA	Recovery	Per cent recovery
γ per ml.	*γ per ml.*	*γ per ml.*	
17.2	48.9	48.2	98.5
34.4	66.1	64.2	97.0
51.6	83.3	81.9	98.0

For the orcinol reaction, dilution with distilled water ranged between 3 and 10 times. For ultraviolet readings, the perchloric acid extracts were diluted 15 to 20 times with 10 per cent perchloric acid. Phosphorus determinations and the diphenylamine reaction were performed on the undiluted extracts. 3 ml. were used for the DNA and 5 ml. for the PNA determinations. The tests were always run in duplicate.

The DNA determination with diphenylamine was also made. All the extraction procedure was performed in the same centrifuge tube in order to avoid any loss and to permit the use of very small samples of tissues.

The indole reaction makes it possible to work with samples as small as 25 mg. of wet tissue (sarcoma 180); when thymus is employed, the amounts may be much smaller. Some recovery experiments were conducted by adding different amounts of pure DNA to the organ extracts. The recovery was complete within the limits of error of the method (±4 per cent) (Table II).

Calculations—In order to compare the results obtained by the various methods, calculations were made according to the following outline. The DNA and PNA contents for 100 γ of dry tissue were calculated from the results of the absorption given by the different reactions on the basis of their specific optical densities (Table I). The per cent of DNA and PNA, multiplied by 0.0989 and by 0.095, respectively, gave the theoretical phosphorus content required by the color reactions for theoretical DNA and PNA. The theoretical absorption in the ultraviolet was also calculated by multiplying the per cent values by the optical densities for 1 γ of DNA or PNA, both at 260 and at 268 mμ.

The sum of the values of PNA and DNA phosphorus and that of PNA and DNA absorption in the ultraviolet, calculated on the basis of the color reactions, were compared with the percentages of phosphorus and of the optical densities found by direct determination.

Materials—Several organs of C57 black mice and of albino rats were studied: liver, kidney, spleen, lung, thymus, and pancreas. The average weight of the animals was 19.5 $\pm$ 0.5 gm. for mice and 150 $\pm$ 5 gm. for rats. Several determinations were also made with sarcoma 180. The values reported are the mean of at least five determinations. All the tests were run in duplicate.

Results

The results obtained are reported in Tables III, IV, and V. All the values refer to the dry weight of the defatted organs. For comparison with the data of the literature, which are often given as per cent of the wet weight, the average per cent of dry weight of the organs studied is also reported.

DISCUSSION

As may be seen from Table III, the values by the indole reaction compare favorably with those by the diphenylamine reaction within the range of the experimental error for both methods. The standard deviation, which is the result of both the individual variations and the experimental error, is sometimes larger with one method, sometimes with the other. The range of error of the two procedures is almost the same, notwithstanding the much smaller amounts of tissue used for the indole reaction.

Some organs, especially spleen, show very important individual variations. This fact is confirmed by data in the literature (7) and has to be kept in mind when comparisons are made between different groups of animals. It may be easily correlated with the very variable histological picture of this organ, sometimes very rich and sometimes poor in germinative centers; furthermore, it is difficult to free the organ from blood to

the same extent in every animal. Thymus also shows rather large individual variations. Smaller variations are found in some other organs, for

TABLE III

Comparison of Determination of DNA and PNA Content of Mouse and Rat Organs

Data expressed as per cent of the dry weight. The standard deviations are the results both of individual variation and error of the methods used.

	Dry weight, per cent of wet weight	DNA, per cent of dry weight		PNA, per cent of dry weight, orcinol	Extreme values	
		Indole	Diphenylamine		DNA (indole)	PNA
Mouse liver	24.0	1.66 ± 0.218	1.71 ± 0.282	4.55 ± 0.298	1.34–1.95	4.05–4.84
" kidney	20.0	2.76 ± 0.155	2.53 ± 0.293	3.01 ± 0.154	2.60–2.98	2.86–3.25
" spleen	20.8	8.32 ± 1.02	8.46 ± 0.488	5.72 ± 0.210	7.30–9.70	5.50–5.98
" lung	20.2	3.73 ± 0.202	3.69 ± 0.311	2.71 ± 0.126	3.44–3.90	2.55–2.86
" thymus	20.8	14.80 ± 1.74	13.80 ± 1.74	5.97 ± 0.222	12.0 – 16.0	5.70–6.24
" pancreas	22.8	2.42 ± 0.336	2.37 ± 0.262	13.95 ± 0.680	1.93–2.66	13.4 – 14.8
Rat liver	23.9	1.15 ± 0.124	1.20 ± 0.136	4.16 ± 0.358	1.02–1.31	3.84–4.77
" kidney	20.0	2.05 ± 0.232	2.12 ± 0.173	2.61 ± 0.082	1.74–2.35	2.55–2.71
" spleen	21.7	3.34 ± 1.08	3.58 ± 1.01	3.66 ± 0.290	2.06–3.68	2.81–4.03
" lung	17.2	3.66 ± 0.164	3.88 ± 0.241	2.75 ± 0.293	3.45–3.85	2.46–3.20
" thymus	17.6	12.12 ± 1.88	12.96 ± 1.02	5.70 ± 0.175	9.30–13.80	5.60–5.90
" pancreas	23.5	1.99 ± 0.175	2.02 ± 0.163	10.64 ± 0.923	1.72–2.15	9.45–12.00
Sarcoma 180	15.4	4.78 ± 0.231		7.75 ± 0.309	4.55–5.25	7.20–8.05

instance kidney and lung. This holds true also for sarcoma 180 when healthy parts of tumors of the same age are chosen.

When comparing the DNA content of organs from mice and rats, one notices a more or less higher content for the former, except for lung. Also PNA is higher in mouse than in rat spleen and pancreas. The data in the literature are very scarce for the mouse; those for the rat are much more abundant. The present data compare favorably with most of the

published data (7). Considerable differences may be found, depending upon the strain, age, and sex of the animals used (7). Therefore animals of the same sex and of similar weight and, for mice, of an inbred strain (C57 black) have been chosen.

Special consideration must be given to comparison among the data obtained from color reactions (indole and orcinol), phosphorus determination, and ultraviolet readings. In Table IV, one observes some differences between the phosphorus calculated on the basis of the color reactions and that determined directly. These are especially marked for lung and

TABLE IV

DNA P and PNA P of Mouse and Rat Organs

Comparison between the dry weight percentages calculated on the basis of the indole and the orcinol reactions with the values found experimentally.

	DNA P, per cent of dry weight (indole)	PNA P, per cent of dry weight	DNA P + PNA P, per cent of dry weight		PNA P / DNA P
			Calculated	Found	
Mouse liver	0.164	0.433	0.597	0.626 ± 0.055	2.64
" kidney	0.273	0.286	0.559	0.537 ± 0.026	1.05
" spleen	0.824	0.543	1.367	1.385 ± 0.077	0.66
" lung	0.369	0.258	0.627	0.582 ± 0.063	0.70
" thymus	1.462	0.567	2.029	1.836 ± 0.274	0.39
" pancreas	0.239	1.325	1.564	1.740 ± 0.090	5.54
Rat liver	0.114	0.395	0.509	0.506 ± 0.052	3.46
" kidney	0.203	0.258	0.461	0.434 ± 0.025	1.27
" spleen	0.330	0.348	0.678	0.623 ± 0.132	1.05
" lung	0.362	0.261	0.623	0.551 ± 0.050	0.72
" thymus	1.200	0.541	1.741	1.600 ± 0.180	0.45
" pancreas	0.197	1.010	1.207	1.240 ± 0.039	5.13
Sarcoma 180	0.474	0.718	1.190	1.140 ± 0.053	1.52

thymus, both of mouse and rat, and for rat spleen. The phosphorus content of these organs is lower than expected; on the contrary, for mouse pancreas it is higher.

Considering the values calculated and those found for ultraviolet absorption, practically the only important difference to be observed is in mouse pancreas, in which the absorption is higher than expected (Table V).

There is greater agreement between the results of the color reactions and the ultraviolet readings than between these determinations and those of phosphorus.

The different ratio of purines to pyrimidines possibly present in the PNA of various organs may explain the lack of agreement when the phos-

phorus and ultraviolet data compare favorably. Only PNA has to be taken into consideration, because DNA has been shown to be very constant in its composition, at least in the same species (8, 9); on the contrary, in PNA the ratio between purines and pyrimidines may vary considerably in different organs (9, 10).

Since the orcinol reaction reveals only the purine-bound ribose, a lower content of purines may demonstrate a lower PNA content than is accounted for by P determination and ultraviolet absorption. On the other

TABLE V

Ultraviolet Absorption Values of Perchloric Acid Extracts of Mouse and Rat Organs

Comparison between the ultraviolet absorption values found experimentally and those calculated from the results of the indole and the orcinol reactions.

	Ultraviolet absorption per 100 γ, dry weight			
	260 mμ		268 mμ	
	Calculated, indole + orcinol	Found	Calculated, indole + orcinol	Found
Mouse liver	0.181	0.187 ± 0.013	0.186	0.187 ± 0.013
" kidney	0.165	0.160 ± 0.015	0.164	0.163 ± 0.015
" spleen	0.400	0.410 ± 0.023	0.400	0.420 ± 0.028
" lung	0.188	0.183 ± 0.014	0.184	0.189 ± 0.015
" thymus	0.580	0.590 ± 0.059	0.590	0.620 ± 0.067
" pancreas	0.484	0.557 ± 0.045	0.465	0.554 ± 0.045
Rat liver	0.154	0.152 ± 0.010	0.149	0.151 ± 0.009
" kidney	0.134	0.125 ± 0.013	0.132	0.131 ± 0.013
" spleen	0.200	0.217 ± 0.025	0.199	0.215 ± 0.029
" lung	0.182	0.173 ± 0.015	0.173	0.176 ± 0.014
" thymus	0.502	0.486 ± 0.049	0.505	0.495 ± 0.051
" pancreas	0.373	0.389 ± 0.031	0.359	0.384 ± 0.033
Sarcoma 180	0.355	0.350 ± 0.016		

hand, if the purine content is higher, the PNA may appear to be higher than that expected from P determination and ultraviolet absorption.

However, when the ultraviolet absorption and the results of the color reactions are in agreement and the P values are lower than expected, the possibility exists, as pointed out for PNA by Gulland (11), that 1 P residue may be bound to 3 instead of to 2 nucleosides. This could account for a lack of as much as one-fourth of the PNA P. This possibility could exist also for DNA, but it has not been proved.

To explain the present results in another way, one should admit the presence in the extracts of some interfering substance (or substances) giving both the color reactions and absorption in the ultraviolet. A substance which interferes with the reaction for DNA with indole has been

found in serum extracts, but it does not show any specific absorption in the ultraviolet. The unknown substance gives a yellow color with indole, which behaves in the same manner that the DNA color does towards chloroform extraction. However, the color produced by serum presents a maximum of absorption at 460 mμ, and the color formed by DNA possesses a maximum at 490 mμ. In addition, the color formed by DNA is completely adsorbed by alumina and that formed by the unknown substance is not, under the selected conditions. Absorption curves of the organ extracts after indole reaction did not differ from the absorption curve given by pure DNA. For this reason the reacting substance of serum does not constitute a limitation for the method of DNA determination in animal tissues. The reacting substance of serum could possibly correspond to the perchloric acid-soluble mucoprotein, which also gives the diphenylamine and the tryptophan perchloric acid reaction (12–16).

The indole reaction for DNA, in the present form, has some limitations, but it is useful when very small quantities of tissue are available.

Comparison among different methods of nucleic acid determination, based on their different components, shows that both sugars and phosphorus determinations yield only relative values, at least as far as PNA is concerned. They may be used alone for comparison of the same organ or tissue. Otherwise, simultaneous study of the ultraviolet absorption is also advisable. The latter seems, at present, to be the best approach to the real value. It is, however, to be noted that this method may produce large variations with small changes in the experimental conditions, as, for instance, pH or heating, and that a variation in the proportion of the different bases could possibly induce variation in the absorption values.

SUMMARY

The application of the color reaction of DNA with indole to the determination of this substance in animal tissues has been studied under different conditions.

An improved method for PNA determination with orcinol and its application to the tissues is also described.

Comparison has been made between the DNA reaction with indole and with diphenylamine.

The total nucleic acid values obtained by the methods proposed have been compared with those from nucleic acid phosphorus determinations and from the ultraviolet absorption.

The limitations of the methods and their validity for direct determination of nucleic acids have been discussed.

I wish to express my appreciation to Dr. C. Chester Stock for his continued interest and aid in the present work, to Dr. L. Cavalieri for kindly

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THE BIOGENESIS OF ALKALOIDS

XIII. THE RÔLE OF ORNITHINE IN THE BIOSYNTHESIS OF STACHYDRINE*

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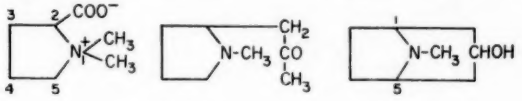
Stachydrine (I), the methyl betaine of proline, occurs in a number of plants (1). Its biogenesis has been the subject of an investigation by Klein and Linser (2) who found an increase in stachydrine content of the plants after an injection of proline into the hollow stems of *Galeopsis ochroleuca* and *Stachys palustris*. They suggest two schemes for the biogenesis of proline, one from ornithine, the other from glutamic acid via pyrrolidonecarboxylic acid.

The metabolic relationship of ornithine, proline, and glutamic acid has been studied in molds (3) and animals (4, 5). The most recent investigations of Stetten with animals (4) and of Fincham with molds (3) indicate that there are two likely pathways linking the three compounds. According to Stetten, the intermediate in the major pathway of proline biogenesis, *i.e.* glutamic semialdehyde, arises mainly by reduction of glutamic acid, and only to a small extent by δ transamination of ornithine. The route from ornithine by α transamination via α-keto-δ-aminovaleric acid is of minor importance in animals. In the higher plants, however, this last route is the only one which has been experimentally demonstrated (6), and our recent work showing that the biogenesis of the pyrrolidine ring present in the tropine part (III) of the alkaloid hyoscyamine proceeds from ornithine (7) strengthens this evidence.

Stachydrine (I) may be regarded as the simplest of the pyrrolidine alkaloids, of which hygrine (II) and cuscohygrine are the most important members. These in turn may be related in structure to the tropane alkaloids with which they occur in *Erythroxylon truxillense* Rusby, and, while some species of *Convolvulus* contain hygrine and cuscohygrine (*C.*



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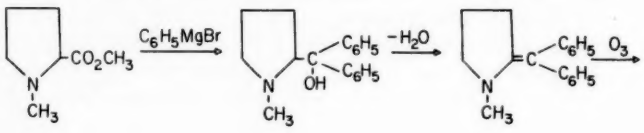
hamadae), others contain tropane bases (*C. pseudocantabricus* Schrenk) (8). It was, therefore, of interest to investigate the relationship of ornithine to stachydrine. At the same time it was hoped that an ambiguity which remained in the biogenesis of hyoscyamine (7) could be removed. This ambiguity arose from the impossibility of differentiating chemically between carbons 1 and 5 in tropine (III). If ornithine were found to be a precursor of stachydrine and it is assumed that the ring closure of the amino acid to the pyrrolidine ring is similar in both the stachydrine and tropine biogeneses, then it would be a reasonable conclusion that the radioactivity should be located on the same carbon in both cases.

The plant selected for feeding ornithine-2-C^{14} was alfalfa (*Medicago sativa* L. Grimm), a plant known to contain stachydrine (9–11). The labeled amino acid was fed to 2 week-old alfalfa seedlings and, after 10 days in contact with the tracer, the plants were harvested and stachydrine was isolated from them as described by Vickery (11, 12), except that the final isolation of the base was effected by means of the Reinecke salt (13) instead of mercuric chloride. The stachydrine thus obtained was not radioactive, and hence in alfalfa stachydrine must proceed from a different source than the pyrrolidine ring of tropine.

The feeding of ornithine-2-C^{14} was expected to give rise to stachydrine with the radioactivity located at carbon 2, and it was assumed that it would be located there exclusively. Since stachydrine is a non-symmetrical molecule, it was possible to work out a method of degradation whereby carbon 2 was isolated. Anhydrous stachydrine when heated above its melting point gave methyl hygrate (IV) (14) which was subjected to the Barbier-Wieland degradation by reaction with phenylmagnesium bromide. The product of the reaction, probably the carbinol V, was dehydrated by being refluxed with a mixture of acetic acid and acetic anhydride.



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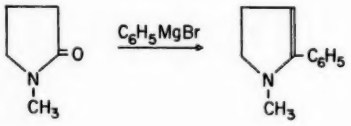

VII VIII

The ethylenic derivative (VI) thus obtained was ozonized and the

ozonide decomposed with dilute sulfuric acid, giving rise to *N*-methyl-2-pyrrolidone (VII) which was caused to react with a further mole of phenylmagnesium bromide. This last reaction, previously described by Craig (15), produced 2-phenyl-*N*-methylpyrroline (VIII) which, when oxidized with aqueous potassium permanganate, gave rise to benzoic acid, the carboxylic group of which contained the carbon originally present in position 2 of stachydrine.

EXPERIMENTAL[1]

Paper Chromatography of Bases Present in Alfalfa—At every step in the isolation of stachydrine from alfalfa (11, 12), the presence of stachydrine was followed by chromatography. As this base occurs with choline in alfalfa, it was important to find a solvent system capable of separating these two bases. Of the many solvent systems tried, the only one suitable was found to be Munier's system Bc20 (16, 17); *i.e.*, the alcoholic layer obtained on shaking together *n*-butanol (100 parts), hydrochloric acid (20 parts, density 1.88), and water (39 parts). It was used on unbuffered Whatman No. 1 paper. With this system the R_F values were 0.41 for choline and 0.52 for stachydrine. The bases were detected by exposing the paper to iodine vapor, which gave brown spots that faded after 48 hours, or, better, by means of Dragendorff's reagent (18) which gave more permanent red-purple spots. In the later stages of the isolation of stachydrine, the reineckate fractions were chromatographed with use of an ethanol-ammonia solvent consisting of 95 parts of 95 per cent ethanol and 5 parts of concentrated ammonium hydroxide. No separation of choline and stachydrine could be effected with this system, the R_F values being 0.45 and 0.40, respectively. But although stachydrine reineckate (and the reineckates of all bases weaker than ammonia) was decomposed by the solvent, stachydrine moving as the free base, choline reineckate (and the reineckates of all bases stronger than ammonia) remained at the origin, and thus a separation was achieved. If choline reineckate was treated with silver nitrate solution, silver reineckate remained at the origin and the free choline moved to its normal position.

Administration of Ornithine-2-C^{14} to Plant—Alfalfa seeds (cultivated variety Grimm) (300 gm.) were dusted with the fungicide Semesan[2] and were then uniformly divided and spread into ten Pyrex trays (21 × 32 cm.) on top of a layer of wetted glass wool. After 3 days in a closed cabinet, the germinating seeds (all of the seeds had germinated) were allowed to continue growing in the open greenhouse, the moisture being kept up by

[1] All melting points are corrected.

[2] Semesan, produced by the Bayer Company, Inc., New York, has for its active principle hydroxymercurichlorophenol.

the addition on alternate days of 50 ml. of water and 50 ml. of nutrient solution (7) to each tray. The nutrient solution was discarded every 7th day, the glass wool washed thoroughly, and fresh solution added. After 14 days, a solution of ornithine-2-C^{14} monohydrochloride prepared by a method previously described (7) (100.4 mg., specific activity of 5.58×10^7 disintegrations per minute per millimole and a total activity of 3.32×10^7 d.p.m. in 100 ml. of water) was fed to the alfalfa (10 ml. per tray) and the plants were allowed to grow in contact with the tracer for 10 days, by which time there was appreciable activity in the leaves (about 2.3×10^3 d.p.m. per gm. of fresh leaves). The total weight of the fresh or dry plant could not be determined, as the plant was inseparable from the glass wool.

Isolation of Stachydrine Hydrochloride—The plants and glass wool were washed with distilled water to remove any adsorbed ornithine and extracted three times successively with 6 liters of boiling water for 24 hours each. The three extracts had the following activities: 2.1×10^6, 0.8×10^6, and 0.4×10^6, or a total of 3.3×10^6 d.p.m., representing 10 per cent of the activity present in the ornithine fed to the plant.

The extracts were combined, concentrated to a volume of 7 liters, treated with lead acetate in the usual way, and further concentrated to 130 ml. This concentrate consisted of a dark brown viscous syrup; it was extracted repeatedly with 95 per cent ethanol. The combined ethanol extract containing all of the material giving a positive Dragendorff test was evaporated to a syrup and dissolved in 1.0 N hydrochloric acid (75 ml.). The bases were precipitated from this solution as reineckate, and the salt was dissolved in acetone and decomposed with silver nitrate as described in the literature (13). The solution containing the bases thus purified was concentrated and made alkaline with ammonia. It had a total activity of 5×10^4 d.p.m. and gave strong spots of choline and stachydrine when subjected to chromatography. Choline was precipitated from this solution with ammonium reineckate, and stachydrine reineckate, m.p. 156–157°, was precipitated by acidification of the liquor with 1.0 N hydrochloric acid. Stachydrine reineckate was converted to the chloride in the usual way. The activity of the crude stachydrine chloride (0.295 gm.) was 8450 d.p.m.

Inactive stachydrine chloride (1.393 gm.) was added to the crude material and the whole recrystallized from absolute ethanol, yielding pure stachydrine chloride (1.215 gm.), m.p. 225° (decomposition), which was completely inactive. The mother liquor from the inactive stachydrine chloride was found to contain all of the activity originally present in this fraction.

To this mother liquor, inactive ornithine monohydrochloride (0.992 gm.) was added. The amino acid salt was reisolated and recrystallized

from aqueous ethanol. It was completely inactive, and, therefore, the activity in the mother liquor was not caused by ornithine-2-C^{14}.

Unknown Bases in Alfalfa—In the course of preparatory work, a 30 gm. sample of alfalfa seeds was grown for 3 weeks and worked up as described by Vickery (11, 12). The material obtained after decomposition of the mercuric chloride precipitate was chromatographed with *n*-butanol-hydrochloric acid (Munier's Bc20 solvent). Development with iodine located five spots of R_F 0.19 (*a*), 0.41 (*b*), 0.49 (*c*), 0.58 (*d*), and 0.73 (*e*). Spots *b*, *c*, and *d* were also detected with Dragendorff's reagent, while Spot *a* gave a blue color with ninhydrin. Spot *b* was identified as choline and Spot *c* as stachydrine. The nature of the other spots was unknown. Spot *d* was not hygric acid (*N*-methylproline, R_F 0.57), since this acid did not give a color with Dragendorff's reagent, nor did it separate completely from stachydrine when a mixture of these two substances was chromatographed, whereas Spot *d* was completely separate from Spot *e*. Nor did Spot *d* represent proline (R_F 0.55), since this amino acid gave a yellow color with ninhydrin and a blue color if the paper had been exposed to ammonia vapor before spraying with ninhydrin. Ornithine (R_F 0.12) gave a purple color with ninhydrin and therefore was not present at Spot *a*. It is possible that one of the spots (*d* or *e*) might be due to betonicine or turicine, the methyl betaines of hydroxyproline which are known to accompany stachydrine in *Betonica officinalis* (19–21). Samples of these, however, were not available for comparison.

Systematic Degradation of Stachydrine to Isolate Carbon 2—Stachydrine was converted to methyl hygrate by the method of King (14). Methyl hygrate (0.812 gm.) dissolved in ether (20 ml.) was added to a solution of phenylmagnesium bromide cooled to −30°, obtained by stirring together magnesium (0.72 gm.), bromobenzene (3.5 ml.), and dry ether (100 ml.). The mixture was subsequently stirred for 20 hours at room temperature. It was then cooled and acidified with dilute hydrochloric acid. The aqueous layer was separated, made alkaline with sodium hydroxide, and extracted with ether. The extract was dried over sodium sulfate and evaporated to dryness. The residue, which crystallized as long needles, consisted of the carbinol (V) (0.635 gm.) which was not further purified but was immediately dehydrated by refluxing for 1 hour with a mixture of acetic acid (15 ml.) and acetic anhydride (15 ml.). Removal of the solvent *in vacuo* left the ethylene compound (VI) as a brown viscous residue which was dissolved in chloroform (20 ml.). Ozone (0.01 mole) was bubbled through the solution, cooled to −30°; dilute sulfuric acid was subsequently added, and the aqueous layer was separated and extracted with chloroform in a continuous extractor. Evaporation of the chloroform from the extract yielded *N*-methylpyrrolidone contaminated with benzophenone. The

mixed product was allowed to react with excess phenylmagnesium bromide and *N*-methyl-2-phenylpyrroline isolated from the mixture by a procedure identical with that used by Craig (15). The compound had the same properties as those described in the literature (15) and gave a picrate, m.p. 141°, and an oxalate, m.p. 138°. *N*-Methyl-2-phenylpyrroline (100 mg.) was refluxed with potassium permanganate (1.0 gm.) in water (100 ml.) for 2 hours, alcohol added to destroy the excess permanganate, and the filtered solution acidified with hydrochloric acid. Extraction with ether yielded benzoic acid, m.p. 119°.

DISCUSSION

Negative results in biochemical work are not always of significance owing to the many inherent variables. Thus the synthesis of stachydrine might occur at a later stage of development of the plant, and the stachydrine isolated might have been material stored in the seeds. Taken at its face value, however, the present result would appear to show that in alfalfa ornithine is not a precursor of either stachydrine or proline if one assumes the facile methylation of proline in the plant. It would indicate that, according to Stetten's metabolic scheme (4), the minor pathways are blocked and that in alfalfa the precursors of proline and therefore of stachydrine are glutamic acid and glutamic semialdehyde. This hypothesis will be tested in these laboratories.

SUMMARY

Alfalfa plants (*Medicago sativa* L. Grimm) were allowed to grow in a nutrient solution containing ornithine-2-C^{14}. After a suitable period of growth, the stachydrine was isolated from the plant and found not to be radioactive.

The significance of the results is discussed in connection with the general problem of the formation of the pyrrolidine ring.

A method of degradation is described whereby carbon 2 of stachydrine can be isolated from the rest of the molecule.

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LIPIDE AND PROTEIN COMPOSITION OF FOUR FRACTIONS ACCOUNTING FOR TOTAL SERUM LIPOPROTEINS*

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The term lipoprotein refers to a group of conjugated proteins in which variable amounts of free and esterified cholesterol, phospholipides, and triglycerides are bound to protein. Practically all of the serum lipides are so bound. Serum lipoproteins have been separated or isolated by precipitation (1–3), electrophoresis (4–6), and centrifugation (7–10). These methods do not yield identical products. For example, precipitation may result in disruption of some of the lipoprotein bonds, and electrophoresis fails to separate lipide-carrying from non-lipide-carrying proteins. The flotation procedure of Pedersen (7), as improved by Lindgren, Gofman, and Elliott (8), is best suited for obtaining quantitative data. In this procedure, advantage is taken of differences in density of the various serum lipoproteins, differences that are the result of variations in the quantity and type of lipide bound to the protein. The actual separation of the lipoproteins from each other, as well as from the non-lipide-carrying proteins, is accomplished by differential centrifugation of serum in media of known density.

Most of the data obtained by the flotation procedure deal only with the levels of a *portion* of the total serum lipoproteins, namely the low density or β-lipoprotein fraction, and provide little or no information on their composition. In the present report, a method is presented in which *all* serum lipoproteins have been separated into four fractions with the following density ranges: Fraction A, less than 1.063; Fraction B, between 1.063 and 1.107; Fraction C, between 1.107 and 1.220; and Fraction D, greater than 1.220. The protein, free cholesterol, cholesterol ester, phospholipide, and triglyceride contents of these four fractions isolated from the sera of man, dog, rabbit, rat, and chicken are reported here.

EXPERIMENTAL

Treatment of Animals—Rabbits and Sprague-Dawley rats were fed Purina laboratory chow. Short comb white Leghorn cockerels were fed Purina

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broiler chow (starter ration). Each dog received a single meal a day, consisting of 25 gm. of horse meat per kilo of body weight, 10 gm. of sucrose, 1 vitamin tablet (11), 1 salt tablet (11), and bone ash. The diets of the human subjects were not controlled.

Collection of Sera—Blood was taken from rats and chickens in the fed state and from rabbits, dogs, and man in the postabsorptive state. Blood was drawn into a syringe coated with silicone, transferred to a centrifuge tube, and allowed to clot. The clot was loosened from the sides of the tube with an applicator stick to aid retraction. The blood was kept at room temperature for at least 30 minutes, then centrifuged at 2000 r.p.m. (1200 $\times$ *g*) for 20 minutes, and the serum obtained was recentrifuged at the same speed for 5 minutes. The serum was separated from the cells as soon as possible and was either analyzed immediately or stored at 4°.

Preparation of Serum Lipoprotein Fractions—In the development of the procedure described here, the ultracentrifuge flotation patterns as determined by Lindgren *et al.* (8) were used as a guide in establishing the densities of the media in which fractionation was finally achieved.[1] All of the so called low density or β-lipoproteins have been included in one fraction, referred to here as Fraction A. The lipoproteins with densities greater than 1.063 were separated into two fractions, B and C, each of which appeared to possess single refractive index boundaries in the analytical ultracentrifuge. Some lipide always remained in the residue (Fraction D) after separation of Fractions A, B, and C. Fraction D contained the serum albumin and globulins, and attempts to isolate a lipoprotein from this fraction by flotation met with no success in our hands.

A model L Spinco ultracentrifuge with a Spinco No. 30.2 rotor was used in the isolation procedure. During centrifugation, the rotor chamber was kept at a temperature of 4°. Since some of the Lusteroid rotor tubes were defective, they were tested before use. This was done by filling the tubes with distilled water and centrifuging them for 1 hour at 30,000 r.p.m. The depression produced in the rotor tubes during testing was not removed before subsequent use.

A 5 ml. aliquot of serum was transferred to each of two Lusteroid rotor tubes, which will be referred to as Tubes I and II. When aliquots smaller than 5 ml. were used, the volume was brought to 5 ml. by the addition of 1 per cent NaCl.[2] The density of the serum in both tubes was then adjusted

[1] We are indebted to Dr. William H. Goldwater of the United States Naval Radiological Defense Laboratory, San Francisco, for determinations of the analytical ultracentrifugal patterns required for the standardization of the separation procedure used here.

[2] The following salt solutions used are expressed as weight per cent at 20°: 1 per cent NaCl, 10.05 gm. of NaCl per liter of solution with a density of 1.005; 5 per cent KBr, 51.83 gm. of KBr per liter of solution with a density of 1.035; 14 per cent KBr,

to 1.107 by adding 4 ml. of 28 per cent KBr. The rotor tubes were capped and the contents mixed by inversion. The level of the meniscus of the diluted serum was marked on the outside of the Lusteroid tubes with a Vaporite brush pen. The tubes were centrifuged for 20 hours at 30,000 r.p.m.

After centrifugation, there was a layer of turbid material (Fractions A and B) at the top of Tubes I and II. This layer usually amounted to about 1 ml. in each tube. The top layers in Tubes I and II were transferred to a single tube (Tube III) calibrated at 4 ml. The transfers were carried out with the aid of a suction device made from small gage polyethylene tubing,[3] with a light placed above Tubes I and II to make the boundaries of the top layers visible. The contents of Tube III were brought to a volume of 4 ml. with 14 per cent KBr, and the density of this mixture was adjusted to 1.063 by the addition of 5 ml. of 5 per cent KBr. The tube was capped, and the contents were mixed by inversion.

The upper part of the inner surfaces of Tubes I and II was wiped with cotton, and 1.5 gm. of solid KBr were added to each tube. Both tubes were immediately shaken briefly, with a circular motion, to keep the KBr from adhering to the bottom, and the total volume of the mixture was made to 9 ml. by the addition of a solution of 14 per cent KBr. The tubes were capped, and the contents mixed by inversion until the KBr dissolved. The density of this solution was 1.220 at 20°. Tubes I, II, and III were then centrifuged at 30,000 r.p.m. for 24 hours.[4]

The second centrifugation effected a separation of the contents of Tube III into a narrow, turbid layer (Fraction A) and a clear lower one (Fraction B). In Tubes I and II, two layers were present, after centrifugation, both clear and with a pronounced boundary between them. The upper one has been designated Fraction C, and the lower one, Fraction D. In chicken and man, Fraction C is yellow in color, probably the result of the presence of bilirubin.

Fraction A was transferred to a 50 ml. centrifuge tube with a short,

155 gm. of KBr per liter of solution with a density of 1.107; 28 per cent KBr, 346.7 gm. of KBr per liter of solution with a density of 1.238.

[3] The suction device was made by inserting a 7 inch length of polyethylene tubing (0.008 inch in diameter) into a No. 0 rubber stopper. This was accomplished by first pushing a No. 17 hypodermic needle through the stopper, then inserting the tubing inside the needle, and, finally, carefully withdrawing the needle. In the same manner, 2 or 3 feet of a larger bore tubing were inserted into the same stopper and attached to a vacuum line. By placing the stopper in rotor Tube II and reducing the pressure by 7 to 8 mm. of Hg, the top layer in Tube I was transferred quantitatively to Tube II through the small gage tubing.

[4] Centrifugation of Fraction C at a density of 1.220 was carried out for various intervals from 12 to 40 hours, and it was found that by 24 hours this fraction was completely concentrated at the top.

conical bottom, by a suction device similar to that described above. Each sample of Fraction C (Tubes I and II) was transferred to a separate 50 ml. centrifuge tube, whereas Fraction B was divided into two parts and each part transferred to a separate 50 ml. tube. Both Fractions D in Tubes I and II were combined in a single 250 ml. Erlenmeyer flask. Since the lower part of Fraction D is viscous, its transfer was facilitated with water washes.

Extraction of Lipides—It is more difficult to extract lipides from isolated lipoproteins (Fractions A, B, and C) than from untreated serum. The large amounts of salt added to adjust the density during the fractionation of serum may account for the change in extractability of lipides. The addition of chloroform to the alcohol-ether mixture generally used for serum extraction improved the recovery of lipides from lipoprotein fractions. It has been suggested that the precipitation of low density lipoproteins with tungstic acid and the subsequent removal of salt improve the extraction of lipides (12). However, we have been unable to obtain quantitative precipitation of low density lipoproteins with tungstic acid or with other protein precipitants.

A sufficient amount of a 6:2:1 mixture of alcohol, ether, and chloroform[5] was added to the tubes containing Fractions A, B, and C to bring the volume in each to 50 ml. The solvent was added forcefully, with a syringe, to insure good mixing. The tubes were heated for 2 hours at 60°, allowed to cool, and then centrifuged for 10 minutes at 2000 r.p.m. (1200 × *g*). The solvent was carefully decanted into volumetric flasks. The extracts from both parts of Fraction B were combined in a single volumetric flask, and the same was done for Fraction C. The protein residue in each tube was washed first with the alcohol-ether-chloroform mixture described above, next with an alcohol-ether (3:1) mixture, and finally with ethyl ether. The centrifuge tubes containing the first washes were heated for at least 15 minutes at 60°. This heating was especially important with Fraction A when the serum was hyperlipemic. The washings of each fraction were combined with its original extract.[6] After the last wash, the tubes were inverted and drained.

15 volumes of alcohol-ether (3:1) were added to Fraction D in the Erlenmeyer flask, and the mixture was heated for 2 hours at 60°. It was then filtered into another Erlenmeyer flask, and the protein and filter paper were washed thoroughly with alcohol-ether. The extract was concentrated

[5] The ethanol used was distilled over KOH pellets. The ethyl ether was distilled over Na_2SO_3 to remove peroxides. The reagent grade chloroform was not treated before use.

[6] Chloroform must be washed out of the protein, since chloroform forms colored complexes with the biuret reagent.

on a steam bath to a small volume and transferred to a 25 or 50 ml. volumetric flask.

Determination of Lipides—Phospholipide (PL) was determined by a combination of the methods of King (13) and Horecker *et al.* (14). Cholesterol was determined by a modification of the Sperry-Brand colorimetric procedure (15). Free cholesterol (FC) was precipitated as the digitonide before determination. Total cholesterol (TC) was determined without precipitation. Total fatty acids (TFA) were determined, after saponification, by a modification of Bragdon's method (16). The lipide constituents determined are expressed in terms of mg. per 100 ml. of serum.

The following empirical conversion factors were used in reporting lipide data: phospholipide = 25 (phospholipide phosphorus); cholesterol esters = 1.67 (TC − FC); triglyceride fatty acids = TFA − (0.667 × PL + 0.67 (TC − FC)); triglycerides = 1.05 (triglyceride fatty acids).

Determination of Protein—The protein contents of the lipide-free residues of Fractions A, B, and C were determined by the biuret method as described by Gornall *et al.* (17). When the protein did not dissolve readily, the mixture containing the biuret reagent was heated for a few minutes. The biuret reagent was standardized by comparison with a protein solution that had been analyzed by the Kjeldahl procedure.

Results

The concentrations of lipides and protein found in the four lipoprotein fractions prepared from the sera of rats, rabbits, chickens, dogs, and man are recorded in Tables I and II. Each serum sample was analyzed in duplicate, and the values obtained on the same serum rarely differed by more than a few per cent. Fractions A, B, and C contained phospholipides, free and esterified cholesterol, and triglycerides. Cholesterol and triglycerides were barely detectable in Fraction D, phospholipide being the major lipide constituent of this fraction. The serum of all species contained more Fraction C protein than Fraction A or Fraction B protein. Since the lipide-protein complex could not be separated from the non-lipide-carrying proteins in Fraction D, the protein content of this fraction was not measured. Except for man, the range of variation for the protein and lipide content of each fraction was quite small. For this reason, only the average values are reported for dog, rabbit, rat, and chicken.

The values for "total" lipoprotein per 100 ml. of serum are not given, but they can be obtained by the addition of the lipoprotein values (Tables I and II) for Fractions A, B, and C. The concentrations of "total" lipoprotein in serum were much greater in man, chicken, and dog than in rat and rabbit. In all species except man, Fraction C constituted 50 per cent

TABLE I

Concentration of Protein and Lipides in Four Lipoprotein Fractions of Five Species

The values are expressed as mg. per fraction per 100 ml. of serum. The value for man is the average of separate determinations on the sera of five subjects. The value for the dog is the average of separate determinations on the sera of three dogs. That for the chicken represents the average of separate estimations on three chicken sera. The value for the rabbit is the average of three determinations, two separate determinations on the serum of two animals and a single determination on a pooled serum sample prepared from the blood of four animals. The value for the rat is the average of three separate determinations on pooled samples obtained from five, seven, and ten rats.

Lipoprotein fraction	Species*	Lipoprotein	Protein	Total lipide	Phospholipide	Free cholesterol	Cholesterol esters	Triglycerides
A	Rat	74	13	61	14	4	11	32
	Rabbit	99	17	82	21	6	19	36
	Chicken	208	35	173	43	15	41	74
	Dog	86	21	65	17	7	9	32
	Man	435	72	363	89	35	128	111
B	Rat	106	35	71	36	7	27	1
	Rabbit	59	25	34	18	3	7	6
	Chicken	60	25	35	17	3	8	7
	Dog	99	35	64	33	6	17	8
	Man	90	30	60	26	6	21	7
C	Rat	234	121	113	55	6	42	10
	Rabbit	277	171	106	57	7	24	18
	Chicken	611	314	297	157	15	68	57
	Dog	758	379	379	200	22	118	39
	Man	399	237	162	79	9	51	23
D	Rat	†	†	40	39	1‡	‡	‡
	Rabbit	†	†	29	28	1‡	‡	‡
	Chicken	†	†	23	22	1‡	‡	‡
	Dog	†	†	20	18	2‡	‡	‡
	Man	†	†	32	29	3‡	‡	‡

* Blood was taken from rabbits 14 hours after they were deprived of food. The dogs received their last meal 20 hours before blood was taken, and the human subjects 14 hours before.

† The values for these components cannot be reported, since Fraction D contained the serum albumin and globulins in addition to lipoproteins.

‡ The concentration of these components was too low to measure. The values in the free cholesterol column are actually for total cholesterol.

or more of the "total" lipoprotein. In some humans, Fraction A was the largest. It is of interest to note that, as the values for Fraction A rose in humans, the corresponding values for Fractions B and C fell (Table II). The concentrations of lipoproteins in Fraction A are about the same in dog and rabbit sera. The serum of the dog, however, contained about 3 times as much Fraction C as did the rabbit.

The percentages of protein and of the various lipides contained in Fractions A, B, and C are recorded in Table III. In all species, Fraction C consisted of about equal amounts of protein and lipide, whereas the lipide content of Fraction A was about 5 times that of protein. In spite of differences in composition among these three fractions, the composition of a

TABLE II

*Concentration of Protein and Lipides in Four Lipoprotein Fractions of Human Serum**

The values are expressed as mg. per fraction per 100 ml. of serum.

Lipoprotein fraction	Subject*	Lipoprotein	Protein	Total lipide	Phospholipide	Free cholesterol	Cholesterol esters	Triglycerides
A	L. A. H.	181	34	147	38	17	57	35
	M. D. S.	319	58	261	70	29	127	35
	J. B.	430	76	354	90	35	114	115
	I. L. C.	590	99	491	116	43	198	134
	C. E.	654	91	563	132	51	144	236
B	L. A. H.	131	38	93	44	9	33	7
	M. D. S.	99	35	64	26	6	21	11
	J. B.	84	32	52	26	5	16	5
	I. L. C.	84	28	56	17	5	26	8
	C. E.	50	19	31	15	4	9	3
C	L. A. H.	486	287	199	92	13	72	22
	M. D. S.	477	278	199	99	11	70	19
	J. B.	438	252	186	98	10	51	27
	I. L. C.	301	183	118	55	5	37	21
	C. E.	293	187	106	51	5	24	26
D	L. A. H.	†	†	28	26	2‡	‡	‡
	M. D. S.	†	†	30	27	3‡	‡	‡
	J. B.	†	†	42	38	4‡	‡	‡
	I. L. C.	†	†	43	29	4‡	‡	‡
	C. E.	†	†	28	25	3‡	‡	‡

* Serum was obtained from adult males 14 hours after the last meal. The ages of the subjects are L. A. H., 27; M. D. S., 26; J. B., 32; I. L. C., 50; and C. E., 39 years.

† See foot-note to Table I.

‡ See foot-note to Table I.

given fraction was similar in all species. For example, the values for phospholipides observed in the five species ranged from 19 to 21 per cent for Fraction A, from 28 to 34 per cent for Fraction B, and from 20 to 26 per cent for Fraction C.

The percentage distribution of each lipide constituent among the four lipoprotein fractions can be calculated from the data of Tables I and II. In all species, Fraction A contained a large portion of the triglycerides. In chicken and dog, the major portion of phospholipides (66 to 75 per cent) was found in Fraction C, while in rat, rabbit, and man this fraction con-

tained from 35 to 46 per cent of the phospholipides. In rat, rabbit, chicken, and dog, Fraction C contained from 35 to 63 per cent of the free cholesterol

TABLE III

Per Cent Composition of Lipoprotein Fractions A, B, and C in Five Species

The lipide and protein constituents of each lipoprotein fraction are expressed as a percentage of the value given for lipoprotein in the third column of Table I.

Lipoprotein fraction	Species	Protein	Total lipide	Phospho-lipide	Free cholesterol	Cholesterol esters	Triglycerides
A	Rat	18	82	19	5	15	43
	Rabbit	17	83	21	6	19	37
	Chicken	17	83	21	7	20	35
	Dog	24	76	20	8	11	37
	Man	17	83	21	8	29	25
B	Rat	33	67	34	7	25	1
	Rabbit	42	58	31	5	12	10
	Chicken	42	58	28	5	13	12
	Dog	35	65	34	6	17	8
	Man	33	67	29	7	23	8
C	Rat	52	48	23	3	18	4
	Rabbit	62	38	21	2	9	6
	Chicken	51	49	26	3	11	9
	Dog	50	50	26	3	16	5
	Man	59	41	20	2	13	6

TABLE IV

Total Cholesterol-Phospholipide Ratio Observed in Four Fractions of Five Species

	Fraction A	Fraction B	Fraction C	Fraction D
Rat*	0.76	0.64	0.56	0.03
Rabbit*	0.83	0.40	0.38	0.04
Chicken*	0.92	0.46	0.35	0.05
Dog*	0.73	0.49	0.47	0.11
Man*	1.28	0.76	0.49	0.10
Subject L. A. H	1.32	0.66	0.60	0.08
" M. D. S	1.50	0.69	0.53	0.11
" J. B	1.16	0.58	0.41	0.11
" I. L. C	1.39	1.23	0.49	0.14
" C. E	1.04	0.63	0.37	0.12

* Average values.

and from 48 to 82 per cent of the cholesterol esters. In man, this fraction contained from 9 to 33 per cent of the free cholesterol and from 13 to 43 per cent of the cholesterol esters. In man, Fraction A contained from 44 to 85 per cent of the free cholesterol and 35 to 82 per cent of the cholesterol ester of the serum.

The cholesterol esters found in Fractions A, B, and C amounted to 162 mg. per 100 ml. of serum in subject L. A. H. and 177 mg. in subject C. E. (Table II). Despite the similarity in the values, the distribution of the cholesterol esters among the three lipoprotein fractions varied considerably. Thus, in C. E. 144 mg. of cholesterol esters were found in Fraction A, whereas in subject L. A. H. this lipide constituent was more evenly distributed among the three lipoprotein fractions.

The ratios for total cholesterol to phospholipide, found for each of the four fractions of the five species, were highest for Fraction A and lowest for Fraction D (Table IV).

DISCUSSION

The analytical ultracentrifuge method of Lindgren *et al.* (8) is well suited for the determination of the homogeneity of lipoproteins. It has also been used for the quantitative determination of individual lipoproteins, but, in calculating the amount of a given lipoprotein from its refractive index pattern, use is made of many empirical factors, some of which are difficult to determine. It should also be noted that measurements based on refractive indices provide no information on the composition of a given lipoprotein. Since flotation procedures rely upon the densities of lipoprotein fractions, it is conceivable that different lipoproteins possess the same density, but differ in composition. Chemical determination of lipoprotein constituents would appear to be the method of choice for the study of lipide transport, since it would provide information on both the composition and the absolute amounts of the lipoproteins. Turner *et al.* (9) have already made use of chemical methods, but their stratification technique failed to separate completely the various lipoproteins from each other or from the non-lipide-carrying proteins.

Turner *et al.* (9) suggested that the high salt concentration used in the flotation procedure might cause a partial degradation of the lipide-protein complexes. In order to explore this possibility, we isolated Fraction A by two methods: (1) directly by flotation at a density of 1.063, and (2) by first separating a fraction at a density of 1.107 and then centrifuging this fraction at a density of 1.063. Both samples of Fraction A were found to have the same protein and lipide content. Thus, the use of different salt concentrations did not alter the composition of Fraction A. When isolated Fraction C was dialyzed to free it of salts and then refractionated, it was recovered with an unaltered lipide and protein content, and no lipide or protein was found in other density segments. These findings, in addition to our failure to detect appreciable amounts of serum lipides in a non-lipoprotein form, lend no support to the view that lipide-protein complexes are degraded by the salt solutions used here for fractionation.

It is of interest to compare the results obtained here for human serum

lipoproteins with those obtained by other investigators. Values for the ratio, (mg. total cholesterol)/(mg. phospholipide), for Fractions A, B, and C and for α_1-, α_2-, and β-lipoproteins are given in Table V. The values for our Fraction C agree closely with those for α_1-lipoproteins as prepared by Russ *et al.* (3), Kunkel and Slater (5), and Nikkila (6). Furthermore, our Fraction A appears to correspond to β-lipoprotein as prepared by four separate groups of investigators. A somewhat similar range of values was reported by Nikkila for α_2-lipoproteins. In view of the differences in the methods used for the preparation of these fractions, the agreement is

TABLE V

Total Cholesterol-Phospholipide Ratio for Lipoprotein Fractions of Human Serum Reported by Various Investigators

Investigator	Method of fractionation	No. of analyses	Lipoprotein fraction	Mg. total cholesterol / Mg. phospholipide
Hillyard *et al.*	Flotation	5	Fraction A	1.28 (1.04–1.50)
" " "	"	5	" B	0.76 (0.58–1.23)
" " "	"	5	" C	0.49 (0.37–0.60)
Oncley *et al.* (2)*	Cohn procedure and flotation	4	β-Lipoprotein	1.05 (0.97–1.32)
Russ *et al.* (3)	Cohn procedure	38	α_1-Lipoprotein	0.51 (0.42–0.62)
" " "	" "	38	β-Lipoprotein	1.35 (1.10–1.53)
Kunkel and Slater (5)	Zone electrophoresis	5	α_1-Lipoprotein	0.50 (0.41–0.62)
" "	" "	5	β-Lipoprotein	1.26 (1.17–1.36)
Nikkila (6)	" "	24	α_1-Lipoprotein	0.63 (0.44–0.87)
"	" "	24	α_2-Lipoprotein	0.77 (0.38–1.10)
"	" "	24	β-Lipoprotein	1.24 (0.97–1.74)

* The phospholipide and cholesterol values reported by these investigators were used to calculate the total cholesterol-phospholipide ratios.

indeed worthy of note, particularly in the case of Fraction A which is not homogeneous.

Oncley *et al.* (2) isolated β-lipoprotein from human serum by purifying a lipide-rich fraction (Cohn III-0) by the flotation procedure and found that it contained 23 per cent protein, 29.3 per cent phospholipide, 8.3 per cent free cholesterol, and 39.1 per cent cholesterol esters. Our human Fraction A contained 17 per cent protein, 21 per cent phospholipide, 8 per cent free cholesterol, 29 per cent cholesterol esters, and 25 per cent triglycerides. Thus, there is a discrepancy between our Fraction A and the β-lipoproteins isolated by Oncley, in regard to their triglyceride contents. This discrepancy can perhaps be explained in part by the observations of Jones *et al.* (18), who stated that their low density lipoprotein classes from S_f 4 to S_f 17 contained little or no triglycerides, whereas their lipoprotein classes

from S_f 17 and higher contained increasing quantities of triglycerides. The S_f 4 to S_f 17 classes may correspond to the β-lipoprotein fraction isolated by Oncley.

The similarity in composition of α_1-lipoprotein of Oncley and Gurd (19) and our Fraction C of human sera suggests that they are the same. Thus the former contained 57 per cent protein, 21 per cent phospholipide, 3.3 per cent free cholesterol, and 15 per cent cholesterol esters, whereas our Fraction C contained 59 per cent protein, 20 per cent phospholipide, 2 per cent free cholesterol, 13 per cent cholesterol esters, and 6 per cent triglycerides.

Lewis *et al.* (10) have made an analytical ultracentrifugal study of the serum lipoproteins of the same five species reported here. These workers found that the high density α_1-lipoprotein was prominent in all five species. Since this fraction is the lipoprotein of the highest density that can be isolated by flotation, it must be identical with our Fraction C, which also was shown here to be present in high concentrations.

Despite wide variations in the levels of the lipoproteins (Table I), the percentage composition of Fractions A, B, and C among the several species (Table III) is remarkably similar. It should also be noted that the rat and rabbit are quite similar with respect to both the percentage composition and levels of each fraction, even though they respond differently to diets high in cholesterol.

Although the four lipoprotein fractions described here cannot be readily identified with lipoproteins studied by others, it is believed that our Fraction A contains β-lipoproteins (Table V) and that Fractions B and C are α_2- and α_1-lipoproteins, respectively (6, 10). Fraction D may contain the high density phospholipide protein reported by Turner *et al.* (20).

SUMMARY

1. Serum was separated, by the ultracentrifuge flotation technique, into four fractions so as to account for total serum lipoproteins. Each fraction was analyzed for protein, phospholipide, free and esterified cholesterol, and triglyceride.

2. The values for the concentration of protein and the various lipide constituents in these four serum fractions are presented for man, dog, rat, rabbit, and chicken.

3. The concentrations of the various lipoprotein fractions varied considerably among the five species studied. However, in four species, dog, rat, rabbit, and chicken, the range of variation in the concentrations of each lipoprotein fraction was quite small. Humans differed from these animals in showing considerable variation in the levels of the lipoprotein fractions in serum.

4. The composition of each lipoprotein fraction with respect to protein and the various lipides was similar for the sera of the five species.

5. The identity of our four lipoprotein fractions is discussed.

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FERRITIN BIOSYNTHESIS

I. CRYSTALLIZATION OF GUINEA PIG FERRITIN*

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The ease of isolation, homogeneity, and unique physicochemical characteristics of ferritin (1) make it attractive for the study of protein biosynthesis. After a preliminary survey, the guinea pig was selected as the species best suited to our investigation, but the established method for the isolation of crystalline ferritin yielded only amorphous ferritin from the tissues of this mammal. Consequently the following investigation of the conditions leading to crystallization of guinea pig ferritin was undertaken. A reproducible method is presented for the isolation of crystalline preparations, as illustrated in Fig. 1.

RESULTS AND DISCUSSION

The method described by Laufberger (2) for horse liver ferritin was applied to the isolation of ferritin from guinea pig liver. The crude solution of ferritin, resulting from the steps in the procedure preceding the addition of $CdSO_4$, was employed in tests of the effect of various factors on crystallization. A portion of each test mixture, prepared on a micro scale, was transferred immediately to a Vaseline-rimmed cover-slip for the preparation of a sealed wet mount in which crystallization could be evaluated microscopically.

Cadmium Sulfate—There was only a limited range of concentration of $CdSO_4$ in which most of the precipitated ferritin was crystalline. It was repeatedly demonstrated, with many different preparations of guinea pig liver ferritin and under a variety of conditions, that the optimal concentration of cadmium ion amounted to only a few millimoles per liter, many times lower than the effective range for horse ferritin. The exact amount depended upon other factors, as described below.

Ammonium Sulfate—It became clear in due course that the amount of ammonium sulfate remaining in the preparation from previous steps in the isolation procedure had an important effect on the cadmium requirement. The more NH_4^+ present, the more cadmium ion was needed, and the more limited was the percentage permissible range in the amount of $CdSO_4$

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added. With ammonium sulfate of 50 mmoles per liter and about 0.5 per cent ferritin concentration, the Cd requirement was about 10 mmoles per liter, and no more than 1 mmole deviation could be tolerated for satisfactory results. In the virtual absence of ammonium ion, the optimal concentration was about 2 mmoles per liter of Cd, and good crystallization with little amorphous precipitation could be obtained between 1 and 8 mmoles per liter. The explanation of this effect possibly lies in the formation of the cadmium-ammonium complex ion. Moderate concentrations of NH_4^+ can be tolerated in the crystallization of horse ferritin, in which the

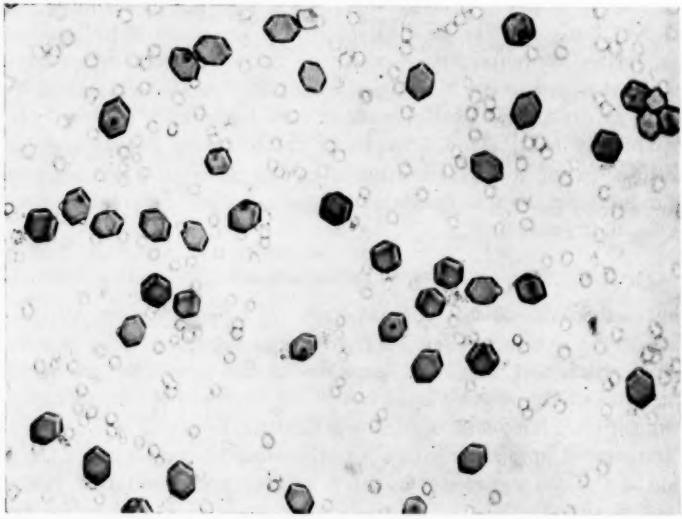

FIG. 1. Crystalline guinea pig ferritin. × 275

cadium ion concentration is high, but with the low levels of cadmium used for guinea pig ferritin the ammonium sulfate level must be carefully controlled. The addition of NaCl at considerable concentration also interfered with crystallization, possibly a non-specific result of ionic strength.

Ferritin Concentration—It has become axiomatic since the work of Northrop, Kunitz, and Herriott (3) that crystallization of proteins is best achieved with concentrated protein solutions. With guinea pig ferritin, however, the more concentrated the protein, other factors being equal, the greater the tendency to form rounded spherites or amorphous precipitate. The optimal concentration found was roughly 0.2 to 0.5 mg. of N per ml.

pH and Buffer Ions—Table I summarizes the result of a series of tests carried out with a fixed concentration of protein, ammonium sulfate, cad-

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mium sulfate, and buffer, but with varying pH and buffer species. The results with the first three and last two buffers suggested a broad optimum of from pH 5.5 to 7. Therefore, in routine isolation of ferritin from guinea pig liver, buffers can be dispensed with, provided the reagents used in high concentration are neutralized to approximately pH 6.

The explanation for the failure to crystallize in the presence of histidine, citrate, and maleate is very likely due to the formation of a cadmium ion complex. This interpretation was supported in the case of histidine by

TABLE I

Effect of pH and Buffers on Crystallization of Guinea Pig Ferritin

Final concentrations, ferritin 2 per cent, cadmium sulfate 5 mmoles per liter, ammonium sulfate 33 mmoles per liter, buffers 67 mmoles per liter. The pH of all buffers was adjusted with HCl or NaOH.

pH	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
Acetate		+	++	+++	++++				
Glycylglycine						++++		0	
Tris*							+		0
Histidine				0	0	0			
Citrate	0				0				
Maleate					0				

pH	4.4	4.8	5.2	5.6	6.0	6.4	6.8
Pyridine	+	+	+	+++			
α-Picoline				+++	++++	++++	+++

Crystallization was rated (by number of plus signs) on the basis of quantity and quality of crystals and absence of amorphous precipitate. Even the best results in this test were not optimal because of too high concentrations of both ammonium sulfate and ferritin.

* Tris(hydroxymethyl)aminomethane.

trying somewhat higher concentrations of $CdSO_4$, which readily yielded crystals in the presence of the previously tested concentration of histidine buffer at pH 6.1.

Temperature—There was a demonstrable temperature effect on the solubility of the guinea pig ferritin crystals in a $CdSO_4$ solution. A solution saturated at 50° yielded crystals upon cooling to 25°. These could be redissolved by warming the suspension and recrystallized by cooling. By slow cooling, crystals measuring 100 × 75 × 50 μ were grown. However, some amorphous material appeared upon redissolving the crystals at 50–60°, suggesting that this otherwise heat-resistant protein may be less so in the presence of $CdSO_4$.

Zinc Sulfate—Since the crystallization of guinea pig ferritin with cadmium was not as satisfactory as that of horse ferritin, the effect of other metal ions was tried. In a limited survey of Zn, Hg, Co, Ni, Mn, Cu, Sr, and Ba, only the first two showed any promise. Of these, mercuric chloride yielded only spherical masses like imperfect crystals, but zinc sulfate yielded typical crystals comparable to those obtained with cadmium sulfate and at comparable metal ion concentrations. However, there was no advantage with zinc over cadmium; in fact, there was a tendency toward more amorphous precipitation and longer delay in crystal formation.

Crystal Shape—The hexagonal crystals reported herein for guinea pig ferritin are somewhat unusual, since ferritin of many species crystallizes as octahedra. This can be seen in the illustrations in Michaelis (4). To those species listed, we can add the rat, which yields crystals indistinguishable in appearance from horse ferritin. The rounded bodies representing guinea pig ferritin included therein resemble the shapes we have frequently obtained with excessive amounts of cadmium. Granick (5) has found human ferritin to form hexagonal crystals, but no details to the method were reported.

According to Granick (1), the octahedral (and tripled octahedral) crystals of horse spleen ferritin were isotropic, whereas the flat, thin square plates obtained with lower cadmium ion concentration were anisotropic. Therefore, our rather flat, hexagonal crystals were examined through crossed polaroids; they proved to be isotropic. We have occasionally observed guinea pig liver ferritin to crystallize in the form of octahedra (mixed with a massive amorphous precipitate) at high Cd concentration or high Zn concentration, and also in the form of thin square plates (at very low Cd concentration).

Guinea pig ferritin, in view of its different behavior toward Cd ion and its different crystal shape, must have distinct structural differences from horse ferritin. It is suggested that the iron storage protein of species reported to be lacking in ferritin might also be induced to crystallize under slightly different conditions.

Procedure for Isolation of Guinea Pig Liver Ferritin

To insure a high ferritin level in the tissue, the guinea pigs were injected intraperitoneally with ferric ammonium citrate amounting to about 40 mg. of Fe per kilo in 2 weeks. A single intravenous dose of saccharated iron oxide served as well (*e.g.* 40 mg. of Fe per kilo). About 500 gm. of liver were ground with an equal volume of water in a Waring blendor, diluted with 3 more volumes of water, and heated at 75° for 5 minutes. The heat-coagulated suspension was centrifuged for 30 minutes at 1000 × *g*. The sediment was resuspended in twice its volume of water and recentrifuged. To the pooled extracts was added an equal volume of $(NH_4)_2SO_4$, 4 moles

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per liter, neutralized to pH 6 with NH_4OH. The mixture was allowed to flocculate and settle in tall cylinders in the cold for 2 days. After the supernatant fluid was siphoned off, the precipitate was further packed by centrifugation, dissolved in an equal volume of water, and dialyzed overnight against water. The solution was adjusted to pH 5.1 ± 0.1 with 1 N acetic acid, the resulting precipitate removed, and the clear solution neutralized to pH 6 ± 0.1.

Tests of optimal $CdSO_4$ level were carried out on a micro scale in the range of 2 to 10 mmoles per liter, final concentration, with a final dilution of the crude ferritin solution of from one-twelfth to one-sixteenth. A combination yielding sharp crystals in maximal yield with minimal amorphous precipitation was used on the bulk preparation, which was then refrigerated for 2 days. The crystals, collected by centrifugation, were suspended in 5 volumes of ammonium sulfate, 0.3 mole per liter, to hasten solution, dialyzed against water with constant agitation until apparently dissolved, and then dialyzed for double this interval. The solution was centrifuged, acidified as above, spun clear, and neutralized to pH 6. Crystallization was then repeated as above, after preliminary testing. After a similar third crystallization, the final crystals were suspended in NaCl, 0.45 mole per liter, dialyzed thoroughly against saline, 0.15 mole per liter, and stored with thymol at refrigerator temperature as an approximately 5 per cent solution. Such solutions were stable for many months.

The yield of recrystallized ferritin from the iron-injected guinea pigs averaged about 40 mg. of protein N per kilo of liver, which was of the order of 30 per cent of the immunochemically determined ferritin content of the livers. The amount of ferritin isolable from liver of iron-treated guinea pigs therefore compares favorably with that obtainable from spleens of normal horses, the richest normal source known.

SUMMARY

Previous methods for the isolation of ferritin, suitable for many species, failed to yield crystalline preparations from the guinea pig. The requisite conditions for crystallization of guinea pig ferritin were determined. These conditions differ from the parent method chiefly in the requirement of much lower concentrations of cadmium ion, ammonium ion, and protein.

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FERRITIN BIOSYNTHESIS

II. ACCELERATION OF SYNTHESIS BY THE ADMINISTRATION OF IRON*

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This investigation was undertaken because of the advantage of employing a specific protein in place of the complex mixture of trichloroacetic acid-precipitable proteins in the study of the mechanism of protein biosynthesis. Ferritin is well suited for such a purpose because of its ease of isolation and unique properties. The production of ferritin is apparently controlled by the amount of iron in the organism. This phenomenon not only provides the investigator with a means of altering the process at will, but is in itself of great general interest, being a special case of the control of protein biosynthesis. The stimulation of ferritin biosynthesis by iron resembles induced enzyme formation and antibody production in that a protein is formed which has a specific affinity for the inducing substance.

A fundamental question concerning the mode of action of iron is whether it accelerates the synthesis of the protein moiety of ferritin[1] or merely retards its breakdown, which would yield the same result if there is normally a continuous turnover. Granick (1) has postulated that iron combines with preexisting apoferritin to form ferritin and that the iron moiety of ferritin protects the protein against breakdown. In this scheme, iron causes an increase in total ferritin by retarding breakdown, and no stimulation of synthesis of the protein moiety need be assumed. Certain experiments of Mazur *et al.* (2) on the properties of ferritin and apoferritin may be construed to support this view. Ferritin was much more resistant to peptic digestion than apoferritin, although further experiments suggested that the protection was actually against acid denaturation, not a very likely hazard in the living cell.

The question was tested in the present investigation by *in vivo* experiments designed to determine, independently of any concurrent ferritin breakdown, the effect of administered iron on the rate of new synthesis of the protein moiety of ferritin. No acceleration of synthesis is predicted in the case of the "retarded breakdown" hypothesis.

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[1] The term ferritin, unless specifically contrasted with the term apoferritin, is meant to include any apoferritin present, and is synonymous with "total ferritin."

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EXPERIMENTAL

Tracer Method—The protein precursor pools of intact guinea pigs were labeled by a single intraperitoneal injection of C^{14}-labeled amino acid, and the initial rates of incorporation of C^{14} into liver ferritin were compared in normal and iron-injected animals. These rates reflect relative rates of synthesis only if the specific activities of the precursor pools are equal. This could not be taken for granted, since the administration of iron could conceivably affect the formation, conversion, or catabolism of amino acids or peptides. However, since all the proteins presumably arise from similar precursors, any such effect of iron should show up in the incorporation of C^{14} into proteins other than ferritin. Therefore, some of these were also analyzed.

4 hours were chosen for the interval between the injection of label and the isolation of ferritin. As indicated by the results, this was a small enough fraction of the turnover time of ferritin to insure estimation of the "initial" rates of incorporation. The incorporation data were therefore not seriously affected by loss of the label due to any concurrent ferritin breakdown.

Immunochemical Isolation of Ferritin—In spite of the crystallizability of ferritin, the chemical isolation of this protein is not ideal for tracer experiments because of incomplete recovery, which permits determination of specific activities, but not of total incorporation. The content of liver ferritin in these experiments varied as much as 4-fold, even within pairs of litter mates. Therefore, specific activity would bear no constant relation to total incorporation and could give no indication of the synthesis rate unless the total quantity present were also known.

A serological isolation offers quantitative recovery for isotope analysis as well as an accurate determination of the amount of ferritin, even on a minute scale. Mazur and Shorr (3) demonstrated the complete precipitation of antigen in the case of horse ferritin and further demonstrated that the iron content does not affect the quantitative antigenic behavior of the ferritin when calculated on a nitrogen basis. Therefore, both ferritin and apoferritin in tissue extracts are included in the specific precipitate.

Antisera—Crystalline guinea pig ferritin for use as immunizing antigen was isolated as described in Paper I (4). Rabbits were hyperimmunized by a series of increasing intravenous doses of alum-precipitated ferritin amounting to 5 mg. of N over a period of a month. The pooled sera, containing about 0.1 mg. of antibody N per ml., were preserved with phenol and merthiolate and calibrated by determining the quantitative precipitin curve (5) with known amounts of crystalline guinea pig ferritin.

Liver Extracts—Weighed samples (1.5 to 2.0 gm.) of tissue were homogenized in a Potter-Elvehjem glass homogenizer with 6 ml. of saline, heated

for 5 minutes at 75°, transferred to a 10 ml. volumetric flask, and made up to volume before centrifuging for 20 minutes at 2000 × *g*. This yielded clear extracts suitable for the precipitin tests.

Precipitin Tests—In view of the variability in content of liver ferritin, a micro scale, semiquantitative, preliminary estimate was performed on each extract so that the precipitin tests could be set up with the assurance of being in the antibody excess zone. The estimate consisted in determining the maximal volume of extract which would still leave detectable excess antibody in the supernatant fluid after precipitation with a portion of serum containing 2 γ of antibody N. The definitive precipitin tests were done on a scale of approximately 50 γ of antibody N and 20 γ of ferritin N at a final volume of 3 ml. in narrow tipped conical centrifuge tubes. The mixtures were incubated ½ hour at 37° and 20 hours at 5°. The precipitates were centrifuged for 10 minutes at 1800 × *g*, which was found adequate for complete sedimentation. They were washed twice by resuspension in 2 ml. of saline at room temperature, which gave the same results as treatment in the cold. Controls for any non-specific precipitation were carried out on the tissue extracts and on the antiserum.

Ferritin C^{14}—The C^{14} content of ferritin was determined by plating and counting the entire specific precipitate in a manner which permitted a micro-Kjeldahl analysis of the same sample. The planchet consisted of a microscope cover-slip, cut in half to fit down the neck of the Kjeldahl flask. During plating and counting, both halves were supported in depressions in an aluminum disk made to fit in the Tracerlab windowless flow counter, model SC-16. The antigen-antibody precipitate was dissolved in 50 μl. of 0.05 N HCl and transferred by a micro pipette to the cover-slip in two layers of small droplets which were dried in a stream of hot air between the application of each layer. Two 50 μl. washes were similarly applied. The self-absorption loss as a function of the mass of protein was determined with samples of C^{14}-labeled plasma protein plated in the manner described. The mass of each specific precipitate, about 0.4 mg., was estimated from the nitrogen determination, and the C^{14} counting data were appropriately corrected. Activities of duplicate washed precipitates, on the average, showed a 5 per cent deviation from the mean.

C^{14} of Mixed Liver Proteins—The heat-coagulated sediment left from the preparation of the liver extract was washed successively by centrifugation and resuspension: 3 times in 5 per cent trichloroacetic acid (heated for 15 minutes at 90° on the second wash), once in alcohol, 3 times in 2:1 alcohol-ether at 60° for 5 minutes, and finally in ether. The dried protein was homogenized in a volatile solvent mixture, plated evenly on aluminum disks, counted with a thin mica window Geiger-Müller counter, and weighed. The specific activity data were corrected for coincidence and self-absorption.

Nitrogen Determination—The cover-slip planchets bearing the antigen-antibody precipitates were broken up in the bulb of the Kjeldahl flasks with a glass rod, boiled 3 hours in 0.5 ml. of H_2SO_4 containing 0.25 per cent SeOCl, and treated terminally with 1 drop of 30 per cent H_2O_2. The ammonia (about 50 γ of N per sample) was steam-distilled for 3 minutes in the Kirk apparatus (6) without immersion of the condenser stem. The distillate collected in boric acid solution was titrated with HCl from a micrometer burette to a bromocresol green and methyl red end-point.

Labeled Amino Acids—DL-Leucine hydrochloride-3-C^{14}, 5.1 μc. per mg., was employed in the first fourteen animals in a dose of either 1 or 2.5 μc. per 100 gm. of body weight. Glycine-2-C^{14}, 14.6 μc. per mg., was employed in the last twelve animals in a dose of either 5 or 10 μc. per 100 gm.

Treatment of Animals—The animals used were immature guinea pigs raised in our colony, which subsisted on Purina rabbit pellets and fresh greens. Each iron-subjected animal was accompanied by a litter mate control of the same sex, except that in each of Litters 11 and 12, one animal served as the control for two iron-injected siblings. Except for iron injection, each control animal was treated the same as its litter mate, but between sets of litter mates the experimental conditions were altered as to age, sex, dose and timing of iron injection, dose of label, and choice of labeled amino acid, as summarized in Table I. All animals were fasted for 24 hours before sacrifice. The iron was given intraperitoneally in the form of ferric ammonium citrate in saline, most often 2 hours before the tracer, and usually in a dose of 1.5 mg. of Fe per kilo (Table I). The labeled amino acid, dissolved in distilled water, was given by the same route 4 hours before sacrifice. The animals were exsanguinated under ether anesthesia. The liver was removed and analyzed for ferritin C^{14}, ferritin N, and mixed liver protein C^{14}, all in duplicate.

Results

Uptake of Amino Acid C^{14} into Ferritin and Mixed Proteins of Liver—The principal result of these experiments is apparent in the bar graphs of Fig. 1, where the C^{14} uptake for ferritin and mixed proteins of liver is plotted for each experimental animal adjacent to its litter mate control. The total incorporation data for ferritin are expressed per gm. of liver in order to render them more comparable for the whole group of animals of varying size. The incorporation data for mixed liver protein are expressed as specific activity (counts per minute per mg. of protein), since the concentration of liver protein in all the animals was assumed to be comparable. The degree of labeling obtained in the liver proteins was directly proportional to the dose of the labeled amino acid employed, as expected with a tracer dose. Therefore, all activity data are expressed per unit dose of label, which was

taken as 1 μc. per 100 gm. of fasted body weight. The data from the experiments with the leucine and glycine are grouped together in the following presentation without any adjustment, for the incorporation per unit dose came out roughly equal for the two tracers. Actually, leucine proved to be the more efficient tracer, since only half of the dose given was in the active L configuration. It should be pointed out that ferritin C^{14} was counted with a windowless counter and mixed proteins were counted with a mica window counter with one-fourth the sensitivity.

TABLE I

Descriptive Data and Treatment of Individual Guinea Pigs

Animal*	Sex	Dose of iron	Iron interval†	Tracer and dose‡	Age at sacrifice	Fasting body weight	Liver weight
		mg. per kg.	*hrs.*	*μc. per 100 gm.*	*days*	*gm.*	*gm.*
1 C	M.	0		L. 1.0	31	267	8.9
1 Fe	"	1.5	12	" 1.0	31	295	8.9
2 C	F.	0		" 1.0	21	224	8.3
2 Fe	"	1.5	12	" 1.0	21	236	8.6
3 C	"	0		" 1.0	31	235	7.3
3 Fe	"	1.5	9	" 1.0	31	229	7.7
4 C	M.	0		" 2.5	25	255	8.1
4 Fe	"	1.5	1	" 2.5	25	249	8.3
5 C	"	0		" 2.5	15	167	5.6
5 Fe	"	1.5	2	" 2.5	15	157	5.5
6 C	"	0		" 2.5	13	147	5.1
6 Fe	"	1.5	2	" 2.5	13	168	6.6
7 C	F.	0		" 2.5	14	177	7.0
7 Fe	"	1.5	2	" 2.5	14	189	6.8
8 C	M.	0		G. 5.0	22	169	5.5
8 Fe	"	1.5	2	" 5.0	22	204	6.1
9 C	"	0		" 5.0	20	207	6.7
9 Fe	"	1.5	2	" 5.0	20	219	7.3
10 C	F.	0		" 5.0	18	179	6.0
10 Fe	"	1.5	2	" 5.0	18	183	6.3
11 C	M.	0		" 10.0	4	74	2.15
11 Fe_1	"	1.5	2	" 10.0	4	95	3.00
11 Fe_2	"	4.5	2	" 10.0	4	89	2.66
12 C	"	0		" 10.0	7	116	3.28
12 Fe_1	"	3.0	2	" 10.0	7	94	2.40
12 Fe_2	"	6.0	2	" 10.0	7	84	2.38

* Each litter bears a number, and the letters C and Fe distinguish between control animals and Fe-treated animals.

† Time between the injections of iron and tracer.

‡ L. represents leucine and G. glycine. The doses listed for glycine should all be multiplied by 1.12 to correct for an error in the original assay of the amino acid.

The tendency for iron to increase the rate of incorporation of C^{14} into ferritin is unequivocally demonstrated, without any comparable effect on

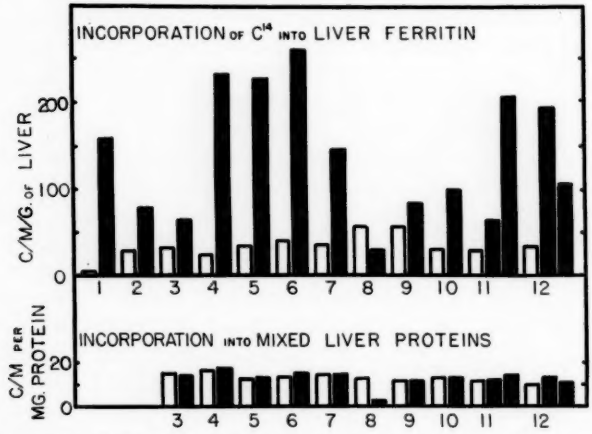
INCORPORATION OF C14 INTO LIVER FERRITIN
C/M/G OF LIVER
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12
INCORPORATION INTO MIXED LIVER PROTEINS
C/M PER MG. PROTEIN
20
0
3 4 5 6 7 8 9 10 11 12

FIG. 1. Incorporation of C^{14} into ferritin and mixed proteins of liver. The empty bars are for control animals and filled bars for iron-injected animals. The numbers designate the litters. The scales of ordinates for the two graphs were arbitrarily adjusted so that the control values for each graph are of approximately the same height.

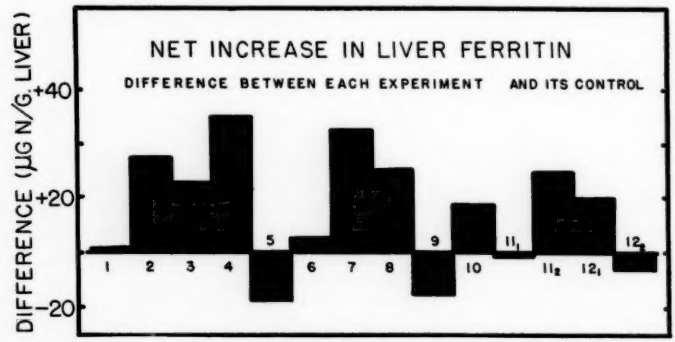
NET INCREASE IN LIVER FERRITIN
DIFFERENCE BETWEEN EACH EXPERIMENT AND ITS CONTROL
DIFFERENCE (μG N/G. LIVER)
+40
+20
−20
1 2 3 4 5 6 7 8 9 10 11₁ 11₂ 12₁ 12₂

FIG. 2. Net increase in liver ferritin. Each bar represents the difference in ferritin content between an iron-treated animal and its own control animal. The numbers identify the litters.

the uptake into general liver proteins. In the case of ferritin, the observed increase of experimental over its own control ranges from 1.5- to 9.5-fold, with a mean of 4.6 ± 0.7 (standard error of the mean). This computation

excludes Litters 1 and 8, for which ratios cannot be assigned because of the indeterminate value for Animal 1 C and the anomalous value in Animal 8 Fe. In the latter case, incorporation into proteins in general was abnormally low, as is seen for mixed liver protein in Fig. 1. The same was true of the plasma proteins, which were also analyzed in twenty of the animals, but not included here because the results were the same as for liver proteins.

Net Increase in Ferritin Content—The concentration of liver ferritin varied enormously from one animal to another, owing presumably to differences in transplacental and intestinal intake of iron. The mean value for the twelve controls was 41 γ of ferritin N per gm. (wet weight), and the difference of each iron-treated animal from its own control is plotted in Fig. 2. There was an average increase of 15.7 γ of N per gm., with a standard error of the mean of 5.74, indicating a probability of less than 0.02 that the increase is not significant. The iron, which was injected on the average only 8 hours before sacrifice, apparently caused an approximately one-third increase in liver ferritin content.

DISCUSSION

Stimulation of Synthesis—The iron injections caused an average 4- or 5-fold acceleration of the incorporation of C^{14} of amino acids into ferritin. The lack of any comparable effect of iron on the incorporation of C^{14} into non-ferritin proteins testifies to the equality of labeling of the precursor pools in the control and experimental groups and therefore permits the conclusion that the incorporation rates reflect synthesis rates. The non-ferritin protein controls further indicate the specificity of action of iron on ferritin synthesis, rather than on protein synthesis in general. It should be emphasized that the net increase in ferritin represents an increase in the protein moiety of ferritin plus apoferritin and not merely a conversion of preexisting apoferritin to ferritin.

The unequal response of the various animals to the injected iron is not clearly correlated with any of the known or controlled variable factors. The lesser incorporation of C^{14} by the females is not statistically significant. As for dosage of iron, it can be assumed that 1.5 mg. of Fe per kilo were sufficient to elicit a maximal response, since the three animals given higher doses did not respond significantly better than average (Table I and Fig. 1). Furthermore, the lower dose was apparently sufficient for a prolonged action, since effects were observed 9 to 12 hours after the injection, judging by the response in the first three litters. This can be explained by the persistence of the injected iron, in keeping with the general failure of the mammalian organism to excrete iron which has once entered.

Synthesis Rates—The rate of ferritin synthesis in the present experiments could be computed by comparing the incorporation rate of C^{14} for ferritin

with that for mixed liver protein, if an independent estimate were available for the rate of liver protein synthesis in fasting young guinea pigs. A rough approximation can be obtained from the turnover time for liver protein of fed adult rats, which has been determined by different laboratories and different techniques to be about 1 week (7–9). This corresponds to a replacement rate of about 1.2 mg. of protein N per gm. of liver per 8 hours, which was the average time interval from iron injection until sacrifice in the present experiments. This value will be used below as an estimate of the synthesis rate for mixed liver proteins in our animals.

The synthesis rates of ferritin and liver protein should be directly proportional to their rates of incorporation of C^{14} if, besides the assumptions heretofore made, we make the reasonable approximation that the amino acid composition of ferritin is the same as that of mixed liver proteins. In order to make the desired comparison of incorporation data, the specific activities of mixed liver protein shown in Fig. 1 were averaged for all animals, converted to total activity on the assumption of 170 mg. of protein per gm. of liver, and raised by a factor of 4 to correct for the sensitivity difference in the counters used for liver protein and for ferritin. The result was 10,000 c.p.m. per gm. of liver. The average value for ferritin in the iron-treated animals was 150 c.p.m. per gm., which, by direct proportion, corresponds to 19 γ of N per gm. of liver per 8 hours, in surprising agreement with the value of 16 γ of N per gm. obtained for net increase. The agreement is consistent with the view that no major proportion of the incorporation is due to "amino acid exchange" under these circumstances. The highest value for ferritin (Animal 6 Fe) was 200 c.p.m. per gm., suggesting that iron-stimulated ferritin synthesis can represent almost 3 per cent of total protein synthesis in the liver, which is considerable in view of the massive serum albumin synthesis.

Turnover—There was a measurable rate of C^{14} incorporation in the control animals, which is consistent with the assumption of a "normal" or "resting" synthesis of ferritin. But in view of the marked stimulation by added iron, it is tempting to postulate that iron is a necessary, as well as sufficient, cause for the synthesis of the protein moiety of ferritin, and that even the observed, resting synthesis is really iron-stimulated. Iron for the purpose is probably ever present, arising from intestinal absorption, from the continuous destruction of red cells, or even from the breakdown of ferritin.

The demonstration of synthesis is no guarantee of turnover; the synthesis could all represent net increase, especially in these growing animals. However, the data of Helwig and Greenberg (10) suggest that liver ferritin turns over even in adult normal guinea pigs. They determined specific activity of chemically isolated ferritin from six animals sacrificed at dif-

ferent intervals after the proteins were labeled by administration of acetate-2-C^{14}. The activity decreased with about a 2 week half life. It is impossible to tell from existing data whether the loss of labeled ferritin occurred at a molecular or cellular level, but, since it did occur, one can speak of turnover. One possibility is the conversion to hemosiderin.

In the present experiments, the incorporation of C^{14} into the liver ferritin of control animals averaged 36 c.p.m. per gm. of liver. Even if all of this synthesis were matched by simultaneous breakdown, the breakdown rate was only about 4 γ of N per gm. per 8 hours, computed as above. Therefore, the maximal rate of net increase in ferritin due to suppression of breakdown alone could be no more than one-fourth of the value actually observed. This supports the conclusion already drawn, that iron increased the rate of synthesis of the protein moiety of ferritin.

Since the average liver ferritin content in the control animals was about 40 γ of N per gm., the turnover time is computed to be at least 80 hours, justifying the earlier assertion that the 4 hour tracer interval was short relative to the turnover time.

The technical assistance of Mr. Henry Chinn in part of this work is gratefully acknowledged.

SUMMARY

The initial rate of incorporation of C^{14} from labeled amino acids into liver ferritin was compared in normal and iron-treated guinea pigs, by means of the quantitative precipitin test for the isolation and analysis of the labeled ferritin. The intraperitoneal administration of 1.5 mg. of Fe per kilo as ferric ammonium citrate produced a sustained, several fold increase in incorporation of labeled carbon from leucine or glycine into liver ferritin. The lack of any comparable effect of iron on uptake of C^{14} into mixed liver proteins, besides demonstrating the specificity of action of the iron on ferritin synthesis, ruled out any interfering effect of iron on protein precursor pools, and therefore permitted the conclusion that the increased uptake of C^{14} indicated accelerated synthesis of ferritin. Ferritin synthesis in some iron-treated animals appeared to account for as much as 3 per cent of the total liver protein synthesis. 8 hours after the injection of iron, there was a demonstrable net increase in the protein moiety of liver ferritin, which roughly agreed in quantity with the amount estimated from the C^{14} incorporation data.

It is concluded that administered iron accelerates the *de novo* synthesis of the protein moiety of total ferritin. Therefore, no hypothesis of retarded breakdown alone is adequate to explain the action of iron.

XUM

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FERRITIN BIOSYNTHESIS

III. APOFERRITIN, THE INITIAL PRODUCT*

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As shown in Paper II (1), the intraperitoneal administration of iron to the guinea pig accelerates the *de novo* synthesis of the protein moiety of ferritin.[1] The present experiments were designed to determine whether the stimulated synthesis involved the iron-free protein, apoferritin, which is known to occur along with ferritin in tissues (2); that is, whether apoferritin is the precursor of ferritin. The reason for questioning such a relationship is as follows. Since the micellar ferric hydroxide moiety of the average sample of crystalline ferritin may constitute 40 per cent of the dry weight, it might be supposed that the incorporation of the iron into the ferritin molecule during synthesis is requisite to the synthesis of the protein moiety. Such an obligatory incorporation of iron as an essential constituent of the protein would provide a simple explanation of its stimulatory action on the synthesis of the protein moiety.

Under such circumstances, any free apoferritin could arise only secondarily, by the release of iron from ferritin. The question of the order of two members in a straight reaction chain can be settled by the determination of their specific activities after the introduction of a radioactive precursor (3). At a sufficiently early interval, the product formed first should have the higher specific activity. But the two products in question, ferritin and apoferritin, are not simply two homogeneous proteins; total ferritin, apparently, consists of a mixture of molecules with the same apoprotein but different iron content. Since the sedimentation constant increases markedly with iron content (2), the ferritin "spectrum" in the present experiments was partially resolved ultracentrifugally into fractions of different iron content.

EXPERIMENTAL

Guinea pigs 6 to 8 weeks old, raised on Pilsbury's "20 per cent rabbit pellets" and fresh chard, were fasted for 24 hours before the experiments.

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† Postdoctoral Research Fellow of the National Institutes of Health, 1951–53.

[1] The term ferritin, unless specifically contrasted with the term apoferritin, is meant to include any apoferritin present, and is synonymous with "total ferritin."

To stimulate ferritin synthesis, the animals were injected intraperitoneally with ferric ammonium citrate in neutral buffered saline, amounting to 0.5 mg. of Fe per kilo. A tracer dose of glycine-2-C^{14} in saline was administered 1 hour later by the same route. The animals were sacrificed after different intervals (Table I).

The liver was homogenized in 2.5 volumes of saline, heated 5 minutes at 75°, and centrifuged for ½ hour at 2000 × *g* to yield a clear extract. The extract was then centrifuged at 100,000 × *g* and divided by a syringe-controlled pipette into upper (top 85 per cent) and lower portions. These were diluted with saline as required and recentrifuged according to the scheme in Fig. 1, dividing the original extract into four fractions.

The total ferritin was serologically isolated from each fraction by duplicate quantitative precipitin tests on a scale of about 30 γ of ferritin N per

TABLE I

Descriptive Data and Treatment of Guinea Pigs

Experiment No.	1	2	3	4	5
Age, *days*	43	49	58	40	51
Sex	M.	M.	F.	F.	M.
Fasted body weight, *gm.*	430	293	405	373	458
Glycine-2-C^{14}, *μc. per 100 gm.*	8.8	10	10	10	10
Tracer interval,* *hrs.*	3.0	1.5	1.5	1.0	0.5

* Time from administration of label until sacrifice of animal.

tube. Ferritin C^{14} and N were determined as described previously (1), and Fe was also determined on the same sample by analyzing an aliquot of the Kjeldahl digest. Selenium was omitted from the digestion mixture (and the digestion was prolonged) to avoid interference with the colorimetric method which was adapted from the plasma iron determination of Kitzes *et al.* (4). The total ferritin from a portion of the original extract was similarly isolated and analyzed.

Results

Distribution of Ferritin among Ultracentrifugal Fractions—The centrifugation times (Table II) were varied somewhat with each experiment on the basis of the results of the preceding one, with the aim of reaching a combination that would give a rather even distribution in all fractions except Fraction IV, in which there were the opportunity and special advantage of attaining higher purity at the expense of yield. For the various fractions, the per cent of the total ferritin nitrogen recovered in all the fractions is an index of the distribution of the protein among the fractions. Over-all

recovery was about 85 per cent, based on the analysis of ferritin nitrogen in the original extracts. The Fe:N ratio in the ferritin of each fraction is an index of the degree of segregation of ferritin molecules of different iron content among the fractions. The fractionation in each experiment can thus be appraised from Table III.

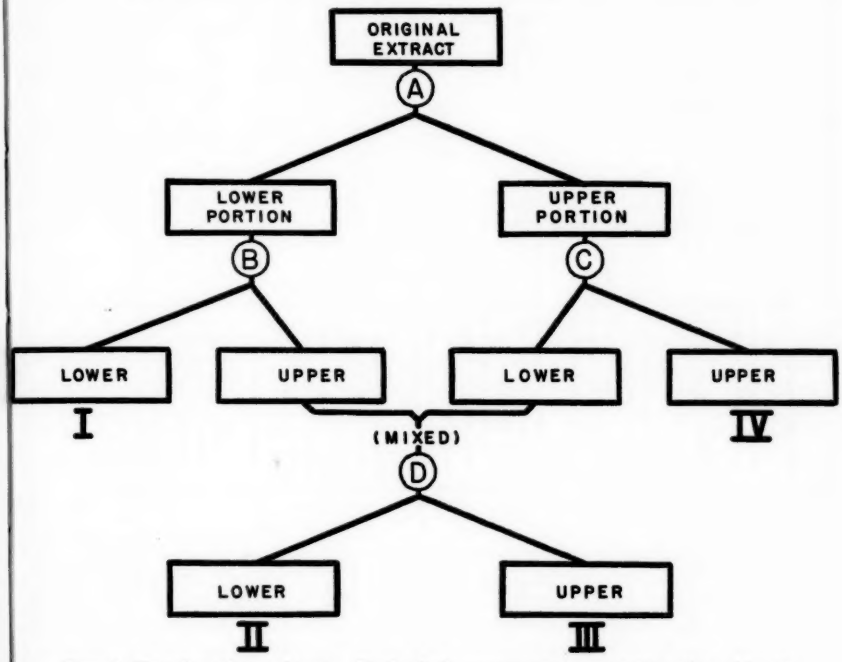

FIG. 1. Fractionation scheme. Each circle represents a centrifugation, identified by letter. In each dichotomy, the fraction on the left is the lower portion, and therefore the richer in "iron-heavy" ferritin. Final fractions are designated by roman numerals.

It is clear from the iron to nitrogen ratios that an appreciable segregation was achieved. The "heaviest" fraction had generally about 4 times the iron content of the "lightest" fraction. In Experiments 4 and 5, about one-eighth of the total recovered ferritin appeared in the apoferritin-rich fraction on a nitrogen basis. In Experiments 2 and 3, about one-fourth appeared in this fraction, which is therefore presumably not as "pure" in apoferritin as in the other experiments, but, on the other hand, the purity of the heaviest fraction was favored.

Specific Activity of Ferritin Fractions—The concentration of isotope in

the ferritin serologically isolated from each ultracentrifugal fraction is plotted in the bar graph, Fig. 2. The highest activity occurred in the

TABLE II

Ultracentrifugal Fractionation

The duration of centrifugation at 100,000 × *g* is given, in minutes, for each centrifugation, designated by letter as in Fig. 1.

Experiment No.*	2	3	4	5
Centrifugation A	20	20	30	30
" B	10	20	40	40
" C	40	60	100	100
" D	15	30	50	50

* See foot-note to Table III.

TABLE III

Distribution of Ferritin in Ultracentrifugal Fractions of Guinea Pig Liver Extract

Experiment No.	Fraction No.*	Per cent of recovered ferritin N	Ratio of Fe:N in ferritin fractions (by weight)
1	1	51	1.4
	2	12	0.4
2	I	10	1.6
	II	24	1.7
	III	39	1.3
	IV	27	0.6
3	I	15	2.2
	II	22	2.3
	III	39	0.8
	IV	23	0.6
4	I	21	1.8
	II	32	1.3
	III	34	0.4
	IV	13	0.2
5	I	25	1.7
	II	34	1.4
	III	30	0.7
	IV	11	0.6

* Fraction numbers designated as in Fig. 1, except for Experiment 1, in which case only two fractions were obtained. Fraction 1 consisted simply of the lower 5 per cent of the extract after a 40 minute centrifugation. Fraction 2 was the upper 50 per cent from a 1 hour centrifugation of a different aliquot of the extract.

apoferritin-rich fraction in each experiment, and the contrast in activities decreased with time. The time factor is critical, because theory predicts an eventual reversal in the relative specific activities of successive compo-

nents in a reaction chain after a single dose of labeled precursor. In the half hour experiment, the activity of Fraction IV was almost 7 times as

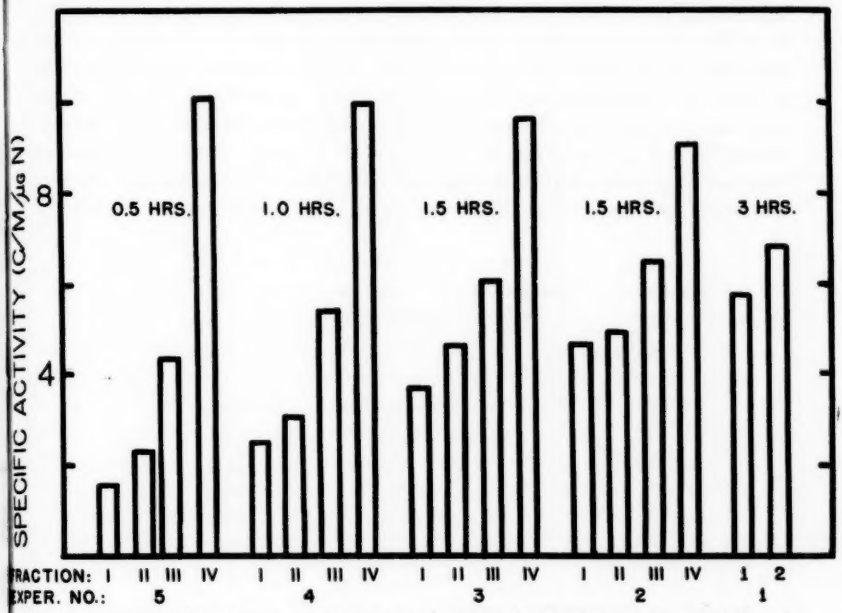
SPECIFIC ACTIVITY (C/M/μg N)
8
4
0.5 HRS.
1.0 HRS.
1.5 HRS.
1.5 HRS.
3 HRS.
FRACTION: I II III IV I II III IV I II III IV I II III IV 1 2
EXPER. NO.: 5 4 3 2 1

FIG. 2. Specific activity of ferritin serologically isolated from ultracentrifugal fractions of guinea pig liver extract. The indicated times are intervals between the administration of radioglycine and sacrifice of the animal. Roman numerals denote the fractions as designated in Fig. 1. In the case of Experiment 1, see the foot-note to Table III. The activities of the fractions within each experiment only are directly comparable. The activity of a given fraction as plotted here cannot be meaningfully compared to that in another experiment because of differences, among other factors, in the quantity of ferritin in the different animals. In Experiment 5, Fraction IV became contaminated to some extent with iron-heavy molecules, owing to a partial tube collapse during fractionation. This was reflected in its Fe:N ratio (Table III). Since it was a case of contamination of iron-poor protein with iron-rich, the proportionate dilution of the original protein by the contaminating protein must have been much lower than the proportionate rise in iron content. Therefore, the effect of the contamination upon the specific activity of the protein was presumed to be minor.

high as that in Fraction I. The ratio of specific activities in the fractions became lower in the longer experiments, until it was hardly above unity in the 3 hour experiment. Consequently, it may be concluded that the observations were made before the expected reversal in specific activities,

that the most active fraction therefore contained the product first formed, and thus that apoferritin preceded ferritin in the sequence.

Time-Course of Incorporation of C^{14} into Ferritin—The appearance of label in *total* liver ferritin as a function of time can be gaged from Table IV, in which the incorporation of C^{14} into total liver ferritin is given in the units previously employed (1). A value from the previous experiments is included for comparison and represents a 4 hour tracer interval. The maximal total incorporation is apparently attained within the first 1 or 2 hours, indicating that by that time the specific activity of the precursor pool had fallen to negligible proportions and no new label was being fed into the apoferritin-ferritin system. The increasing specific activity of the "iron-

TABLE IV

Time-Course of Incorporation of C^{14} from Glycine into Liver Ferritin in Iron-Treated Guinea Pigs

Tracer interval*	Total incorporation
hrs.	*c.p.m. per gm. liver per unit dose†*
0.5	39
1.0	71
1.5	118‡
3.0	112
4.0	125§

* Time between injection of label and sacrifice of animal.

† Unit dose of 11.2 μc. of glycine-2-C^{14} per 100 gm.

‡ Average for Experiments 2 and 3.

§ Average for the six iron-injected, radioglycine-treated animals among those previously reported (1).

heavy" ferritin at the expense of the lighter (Fig. 2) reflects the continuing conversion of the early labeled apoferritin to ferritin.

DISCUSSION

Even in iron-stimulated ferritin synthesis, the results demonstrate that the iron, at least in large quantities, is not attached to the product until the apoferritin is completed. The data are consistent with the hypothesis that essentially iron-free apoferritin is indeed the initial product. Under such circumstances, if one is to conceive of iron as directly affecting the synthesis mechanism for this protein, it must not be in the simple stoichiometric manner postulated in the introduction; the ferric hydroxide micelles cannot be considered an essential constituent. If iron acts directly, it must do so in some catalytic rôle. One such conceivable mechanism, which would provide an explanation of the specificity of action of iron, would be

that the specific ferric hydroxide polymer characteristic of ferritin (5) acts as part of a template in the apoferritin synthesis apparatus, where it could influence the configuration of the peptide chains forming the iron-binding sites of the molecule.

It would appear, however, that before assuming a *direct* action of iron on apoferritin synthesis, one simple hypothesis for an indirect mode of action remains to be disproved. This would depend solely on the conversion of apoferritin to ferritin by iron, as first postulated by Granick (6). But, whereas the accumulation of ferritin protein moiety in such a scheme cannot be explained by protection against breakdown (1), it could be explained if apoferritin were an inhibitor of its own synthesis. Removal of apoferritin as ferritin would relieve this inhibition. The inhibition can be conceived of as analogous to the commonly observed inhibition of enzyme-catalyzed reactions by the products of the reaction. It is necessary to assume here that the apoferritin interferes with the protein-synthesizing system; for example, by competitive inhibition at the site of synthesis. In the simple concept of mass action, uncomplicated by the presence of a rate-limiting amount of catalyst, removal of apoferritin could "drive the reaction to the right" only by slowing the reverse reaction. This would not account for the observed acceleration of the forward reaction, as evidenced by the increased rate of incorporation of label from precursors (1). This proposal is currently being tested experimentally.

The technical assistance of Mr. James Peters in portions of the above work is gratefully acknowledged.

SUMMARY

Tracer experiments in guinea pigs were designed to indicate which, ferritin or apoferritin, was the prior product in iron-stimulated ferritin synthesis. After a tracer dose of glycine-2-C^{14}, liver ferritin was fractionated ultracentrifugally and the fractions analyzed for C^{14}, N, and Fe by the quantitative precipitin test. In the shorter term experiments, the apoferritin-rich fraction was by far the most active, which is consistent with the hypothesis that iron stimulates production of the iron-free protein.

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THE DIGESTION IN VITRO OF TRIGLYCERIDES BY PANCREATIC LIPASE

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The formation of partial glycerides by the hydrolysis *in vitro* of triglycerides with pancreatic lipase was first reported by Artom and Reale (1) and then again several years later by Frazer and Sammons (2). In these studies apparently no attention was paid to the isomeric form of the digestion products. Desnuelle *et al.* (3) further studied the hydrolysis of triglycerides and showed it to be a series of stepwise reactions from triglyceride to diglyceride to monoglyceride to glycerol, fatty acid being released at each step. The quantitative aspects of this work were weakened by their supposition that the hydrolysis was a random one. The pertinent literature in this field has been the subject of an excellent review by Desnuelle (4).

The hydrolysis *in vivo* of triglycerides was reported by this laboratory (5) to be a directed one from triglyceride to 1,2-diglyceride to 2-monoglyceride. Partial conversion of 2-monoglyceride to the 1 isomer also appeared to occur in the intestine. It was likewise reported that at the conclusion of hydrolysis *in vitro* only the 1 isomer of monoglyceride was present. Subsequently, Borgström (6), using methods never previously applied to these types of materials, reported that there is selective formation of 1,2-diglycerides and 2-monoglycerides when triglycerides are hydrolyzed *in vitro* with pancreatic lipase.

In the studies reported here the course of hydrolysis *in vitro* was found to be the same as hydrolysis *in vivo*. From some of the characteristics of the reactions, it appears that lipase may be specific for the hydrolytic and esterification reactions occurring at the primary hydroxyl group positions of glycerol.

Methods

The digestion conditions used in these studies were essentially those described by Desnuelle *et al.* (3), except that the buffer concentration was increased to 1.0 M. Unless otherwise indicated, the digestion mixture consisted of 13.6 ml. of 1.0 M ammonium chloride-ammonium hydroxide buffer adjusted to pH 8.0, 0.3 ml. of a 0.15 per cent aqueous solution of bile salts (Difco), 1.0 ml. of a 45 per cent aqueous calcium chloride solution, 2.3 ml. of an aqueous suspension of 156 mg. of steapsin (Difco), and 2.0 gm. of

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triglyceride. In all of the studies but one, "winterized" cottonseed oil[1] was the substrate used. During the digestion period (15 minutes) at 40° ± 0.5°, the mixture was continuously agitated and a pH of 8.0 was maintained by the addition of 4 M ammonium hydroxide.

In the studies on the effect of electrolyte concentration, the buffer was omitted, but otherwise the standard digestion procedure given above was used. Although the digests initially contained known concentrations of electrolyte, the addition of ammonium hydroxide to maintain the pH necessarily resulted in a dilution of the electrolyte. The more rapid the rate of hydrolysis, the greater the quantity of ammonium hydroxide that was needed, and hence the greater was the dilution of the electrolyte. To allow for this dilution, the amount of added ammonium hydroxide was measured and the final molar concentration of the electrolyte was calculated. From the initial and final molarity, the "average" molarity was calculated and this latter figure was used as a basis for comparison.

At the end of the digestion period the pH was adjusted to 2 with N HCl, 25 ml. of ethyl alcohol were added, and the lipides were extracted with ethyl ether. The ethyl ether solution was washed with water and the lipides were treated in the same manner as in the studies *in vivo* (5). The extracted aqueous layer, to which were added the water washings of the ethyl ether solutions, was retained for glycerol analysis.

The free fatty acid content of the lipides was determined by titration. 1- and 2-monoglycerides were determined by the isomerization and analytical procedures described by Martin (7). Free glycerol was determined by the method of Lambert and Neish (8). Microadaptations of the neutralization equivalent technique, the Wijs iodine value procedure, and the hydroxyl value method of the American Oil Chemists' Society were used (9).

In certain of the studies fractionation of the lipides was carried out. The free fatty acids were isolated first with Amberlite IRA-400, according to the procedure described by Cason *et al.* (10). The method of "completion of squares" (11), by solvent extraction (multistage separatory funnel separation), was employed for the isolation of monoglycerides and diglycerides. For this purpose, the lipides of the digest, from which the free fatty acids had been removed by Amberlite treatment, were passed through a 4 × 4 "completion of squares" process with 80 per cent ethanol-Skellysolve F as the solvent pair. Under these conditions the monoglycerides will be found in alcohol Fractions 3 and 4. The lipides in the Skellysolve Fraction 1, which are essentially all of the diglycerides and triglycerides, were then distributed in a 10 × 10 "completion of squares" process with 95 per cent

[1] Cottonseed oil from which the higher melting glycerides have been removed by cooling and filtration of the liquid from the solid portions.

methanol and isooctane as the solvent pair. In this system the diglycerides will be found chiefly in alcohol Fractions 4, 5, 6, and 7.[2]

Results

Type of Mono- and Diglyceride Isomers Formed

In the portion of the studies carried out *in vitro* that was referred to in the earlier report (5), digestion periods of 60 minutes or more were used. Subsequently, digests carried out for shorter periods of time have been

TABLE I

Proportion of Isomers of Monoglyceride Found after Various Periods of Hydrolysis of Triglycerides

Length of digestion	1-Monoglyceride	2-Monoglyceride	Total monoglyceride	Per cent as 2-monoglyceride
min.	*weight per cent*	*weight per cent*	*weight per cent*	
15	8.1	8.7	16.8	52
30	15.7	10.1	25.8	39
60	28.1	3.4	31.5	11

TABLE II

Digestion Products of 2-Oleoyl Dipalmitin

	Weight per cent
Free fatty acid	34
Monoglyceride*	17
Diglyceride	33
Triglyceride	16

* Consisting of 25 per cent 1-monoglyceride and 75 per cent 2-monoglyceride.

analyzed for 1- and 2-monoglycerides. The results of one such series are given in Table I. Of the monoglycerides found at the end of 15, 30, and 60 minute digestion periods, 52, 39, and 11 per cent, respectively, were the 2 isomer. The decrease in the relative proportions of 2-monoglycerides with time is probably due to isomerization rather than to initial formation of 1-monoglycerides, as indicated below in studies with 2-oleoyl dipalmitin as a substrate.

In order to check further on the formation of 2-monoglycerides, and to investigate the type of diglycerides formed, digestion of 30 minutes dura-

[2] The authors wish to acknowledge the assistance of C. B. Stewart, of these laboratories, who developed these particular solvent separation methods.

tion was carried out with 2-oleoyl dipalmitin as the substrate. The composition of the lipides at the end of the digestion is given in Table II.

From this lipide mixture the free fatty acids, monoglycerides, and diglycerides were isolated. Since the initial substrate had a known glyceride composition, it was possible to calculate what fatty acids, monoglycerides, and diglycerides could be formed by the various theoretical routes of splitting. The values for the possible products and those found for the fractions isolated are given in Table III. From these data it can be seen that es-

TABLE III

Characteristics of Observed and Theoretical Digestion Products of 2-Oleoyl Dipalmitin

	Purity	Iodine value	Mol. wt.
Free fatty acid fraction			
	per cent		
Observed	100	8.7	263
Palmitic*		0	256
Oleic*		90	282
Monoglyceride fraction			
Observed	88	61	
Monoolein*		71	
Monopalmitin*		0	
Diglyceride fraction			
Observed	89	44	
Oleoyl palmitin*		43	
Dipalmitin*		0	

* Theoretical values.

sentially all of the free fatty acids were palmitic acid, the monoglycerides were monoolein, and the diglycerides were oleoyl palmitin. Thus, the course of hydrolysis is apparently 2-oleoyl diplamitin → 2-oleoyl-1-monopalmitin → 2-monoolein, palmitic acid being released at each step. Since the monoglyceride fraction consisted essentially of monoolein, it would appear that the 1-monoglyceride present in the sample arose from the isomerization of 2-monoolein to 1-monoolein.

Factors Influencing Course of Digestion

The effect of varying the constituents of the digest was investigated next. For ease of analysis in these studies the amounts of monoglyceride and of free fatty acid formed were used as criteria of the rate of hydrolysis.

Electrolyte Concentration—In the first series of studies the effect of the electrolyte concentration on the rate of digestion was considered. The amounts of monoglyceride found after 15 minutes of hydrolysis, when the digest contained various concentrations of ammonium chloride, ammonium sulfate, sodium chloride, or glucose, are shown in Fig. 1. In the absence of electrolyte the digestion proceeded quite slowly. As the concentration of electrolyte was increased, the rate of digestion passed through a maximum, then fell off with further increasing concentration. On the other hand, the addition of a non-electrolyte, glucose, had no effect on the rate of hydrolysis. Although not shown, the formation of free fatty acid followed a similar pattern.

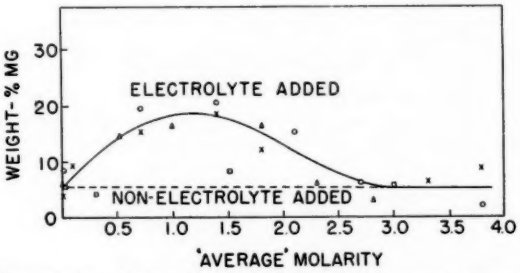

FIG. 1. Effect of electrolyte and non-electrolyte concentrations on the enzymatic formation of monoglycerides (*MG*) from triglycerides. ×, NH_4Cl; ○, NaCl; △, $(NH_4)_2SO_4$; □, glucose.

Bile Salt Concentration—The effect of varying the concentration of bile salts on the rate of digestion, as measured by the amount of monoglyceride and free fatty acid formed, is shown in Fig. 2. When 10 mg. of bile salts (0.05 per cent concentration) were added, the maximal rate of activity was obtained. At higher or lower concentrations the rate of hydrolysis was slower.

Enzyme Concentration—The effect of varying the amount of enzyme in the digest on the rate of monoglyceride and free fatty acid formation is shown in Fig. 3. As the amount of added enzyme increased, so did the amount of digestion products formed. However, the response was not linear but in the form of S-shaped curves.

Kinetics of Reaction

In the final series of studies, digestion periods of 5, 10, 15, 20, and 30 minutes were employed. The concentrations in moles per cent of the various glyceride types at the end of these periods are shown in Fig. 4. At the end of 30 minutes of hydrolysis no free glycerol was present.

The glyceride curves are the type that would be expected for a series of consecutive reactions. A plot of the length of time of hydrolysis against

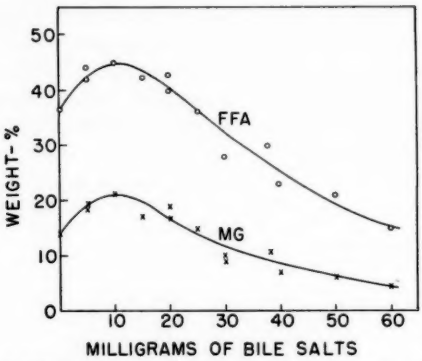

FIG. 2. Effect of bile salt concentration on the enzymatic formation of monoglycerides (*MG*) and free fatty acids (*FFA*) from triglycerides.

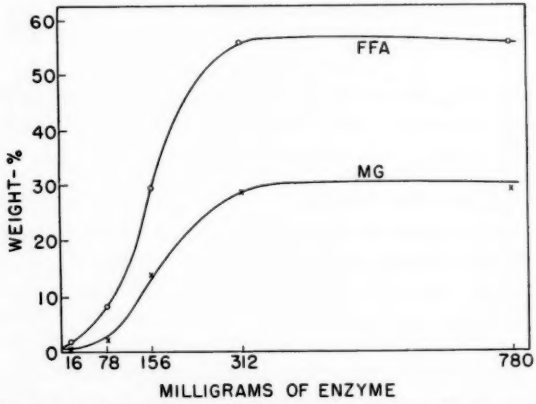

FIG. 3. Effect of enzyme concentration on the enzymatic formation of monoglycerides (*MG*) and free fatty acids (*FFA*) from triglycerides.

the log of moles of unhydrolyzed triglyceride gives a straight line, indicating the hydrolysis of triglyceride to diglyceride to be a pseudomonomolecular reaction. The rate constant was calculated to be 0.07 min.$^{-1}$.

Since the amount of monoglyceride formed is equal to the amount of diglyceride hydrolyzed, and as there was no appreciable monoglyceride hydrolysis, the monoglyceride values can be used in determining the rate

of diglyceride hydrolysis. During the initial period of digestion, 0 to 5 minutes, the rate of formation of monoglyceride, and hence of diglyceride hydrolysis, was 1.2 moles per cent per minute. This rate increased to 2 moles per cent per minute during the second 5 minutes of hydrolysis and remained relatively constant at this rate for the remainder of the time intervals studied. This constancy was obtained in spite of wide variations in the concentration of diglyceride during the course of the hydrolysis. Thus, the conversion of diglyceride to monoglyceride is indicated to be a zero order reaction having a velocity constant of approximately 2 moles per cent per minute.

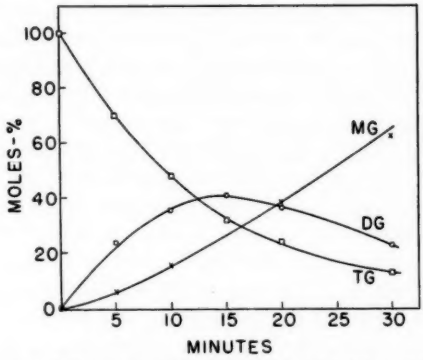

FIG. 4. The course of the enzymatic hydrolysis of triglycerides. *MG* = monoglyceride, *DG* = diglyceride, and *TG* = triglyceride.

Attempts thus far made to determine the velocity constant for the reaction monoglyceride to glycerol have been unsuccessful. At the end of a 90 minute hydrolysis period, with triglyceride as the original substrate, the concentration of monoglyceride is still increasing and only 10 per cent of the glycerol present has been hydrolyzed to free glycerol. Apparently under these conditions the last step in the hydrolysis proceeds at a very slow rate.

DISCUSSION

The reason for the failure to recognize the formation of 2-monoglycerides in our earlier experiments was the use of hydrolysis periods of 60 minutes and longer. When shorter digestion periods were employed, as in these studies, considerable quantities of the 2 isomer were found. Although the monoglyceride initially formed is the 2 isomer, with longer exposure to these digestion conditions there is a proportionate increase in isomerization of 2-monoglyceride to 1-monoglyceride. The value of 11 per cent for the

amount of the 2 isomer present after 60 minutes of hydrolysis is approximately that for an equilibrium mixture of 1- and 2-monoglycerides (7).

The results of the studies both *in vitro* and *in vivo* still do not make it clear whether the conversion of 2-monoglyceride to the 1 isomer is solely enzymatic or merely the result of physical and chemical conditions present during digestion. In the process of isolating the lipides isomerization could also occur, even though all efforts were made to minimize this possibility. Thus, the ratio of 1-monoglyceride to 2-monoglyceride at the completion of the hydrolysis period remains uncertain.

The results obtained when 2-oleoyl dipalmitin was used as a substrate further demonstrate that the hydrolysis of triglyceride is a directed one from triglyceride to 1,2-diglyceride to 2-monoglyceride. The figures obtained were not in absolute agreement with theoretical values, but these minor differences can be attributed to failure to isolate entirely pure fractions and to the possibility that esterification is taking place at the same time as hydrolysis. Borgström (12) has shown that esterification does occur both *in vitro* and *in vivo*. However, the presence of calcium ions in the experiments reported here should keep any synthesis involving free fatty acids to a minimum, since these acids were held in an insoluble state as the calcium soaps.

A similar course of digestion for glycerides of short chain fatty acids has been reported by Schonheyder and Volqvartz (13). Using simple monoglycerides, diglycerides, or triglycerides of propionic acid as substrates, they concluded that the hydrolysis of tripropionin was selectively to 1,2-dipropionin and then to 2-monopropionin which in turn was hydrolyzed very slowly. This route of hydrolysis was observed with both pancreatic lipase and liver esterase.

In the experiments reported here, in which the glycerol was esterified with long chain fatty acids, a selective formation of 1,2-diglycerides and 2-monoglycerides was also found. Similar results with respect to 2-monoglyceride formation have been obtained *in vivo* with triglycerides consisting of short (14) and long (5) chain fatty acids. These results indicate that pancreatic lipase is specific for the esterified primary hydroxyl groups of glycerol.

If the enzyme is specific for the primary hydroxyl groups, any 1-monoglyceride that is present should also be hydrolyzed. Thus, there is some indication that little isomerization of 2-monoglycerides takes place under the digestion conditions. On the other hand, the considerably different physical properties of the monoglycerides, *e.g.* solubility, interfacial orientation, etc., as compared to those of diglycerides and triglycerides, could account for the extremely slow rate of hydrolysis of the monoglyceride.

Consideration of the reactions triglyceride to diglyceride and diglyceride

to monoglyceride would lead one to expect that both would be pseudomonomolecular reactions. The experimental values showed this to be true for the hydrolysis of triglycerides. However, the conversion of diglyceride to monoglyceride was found to be a zero order reaction during all but the initial period of hydrolysis. During a portion of the first 5 minutes of hydrolysis the reaction may have been first order. As the reaction proceeded, some other factor became rate-limiting and hence the change in order of reaction.

The change in reaction order of the diglyceride to monoglyceride reaction also would be expected to occur for the reaction triglyceride to diglyceride, if both were catalyzed by the same enzyme. However, the rate of triglyceride hydrolysis did not change over the course of the digestion. Thus, the possibility is indicated that two separate enzymes may be involved in the hydrolysis of triglycerides and diglycerides.

The unequivocal establishment of the kinetics of the hydrolysis of various isomers of diglycerides and monoglycerides and the effect of the surface activity of monoglycerides on hydrolysis can be determined only by using pure 1,2- and 1,3-diglycerides and 1- and 2-monoglycerides as substrates. Such studies are currently being carried out in our laboratory.

In most of these studies, the initial buffer concentration of 1 M was based on the results of preliminary experiments, indicating that such a high concentration of buffer gave a fast rate of reaction. The experiments summarized in Fig. 1 demonstrate that this effect was a function of the electrolyte concentration and did not depend on the buffering action of the ammonium chloride. The fastest rate of hydrolysis was obtained with an initial electrolyte concentration of 2 M, which under the conditions described would give a final "average" molarity of 1.4. These observations may be a reflection of the effect of the electrolyte concentration on the physical properties of the enzyme, such as solubility or the extent of unfolding of the protein molecule with resulting changes in available reaction sites.

The results obtained when various concentrations of bile salts were used present two interesting points: The first is that even in the absence of bile salts hydrolysis proceeds quite rapidly and the rate is only slightly increased by their presence. The second is that hydrolysis is inhibited by higher concentrations of bile salts.

Under the conditions of these experiments bile salts appear to have a minor rôle in the digestion of glycerides. In this connection it is interesting to note the wide variety of effects obtained by Wills (15) in his studies on the effect of various detergents and bile salts on the rate of hydrolysis by pancreatic lipase. Borgström (16) has suggested that one function of the bile salts *in vivo* may be to lower the pH optimum of lipase so that it will more closely correspond with that in the intestinal tract.

It will be noted that when the concentration of enzyme was varied the initial portion of the curve was not linear as would normally be expected. However, it should be recognized that the hydrolysis is not a single reaction but a series of consecutive reactions. In these experiments the amounts of monoglyceride and free fatty acid formed were used as the criteria of the rate of hydrolysis. The initial lag in these curves can therefore be attributed to the reaction at low enzyme concentration being predominantly triglyceride to diglyceride. When viewed in this light, these are the type of curves that should be obtained under these conditions.

SUMMARY

The nature of the products and some of the factors influencing the hydrolysis of triglycerides by pancreatic lipase *in vitro* have been studied. It was found that (*a*) hydrolysis was favored by a high electrolyte concentration (1 to 2 M), (*b*) bile salts at low concentration caused a slight increase in the rate of hydrolysis but at higher levels were inhibitory, (*c*) increasing the amount of enzyme resulted in the formation of more digestion products, but the effect was not linear.

Digests of less than 60 minutes duration contained appreciable quantities of 2-monoglyceride, as well as 1-monoglyceride. If hydrolysis was allowed to proceed for 60 minutes, only the equilibrium mixture of 1- and 2-monoglyceride was present.

When 2-oleoyl dipalmitin was used as a substrate, the diglyceride formed was oleoyl palmitin and the monoglyceride was monoolein. Thus, the course of hydrolysis of triglycerides *in vitro* appears to be the same as that *in vivo*, namely, a series of directed stepwise reactions from triglyceride to 1,2-diglyceride to 2-monoglyceride. The 1-monoglyceride is the result of isomerization of the 2-monoglyceride.

The hydrolysis of triglyceride to diglyceride was found to be a first order reaction with a velocity constant of 0.07 min.$^{-1}$. The conversion of diglyceride to monoglyceride was chiefly a zero order reaction with a velocity constant of 2 moles per cent per minute. The hydrolysis of monoglyceride to glycerol proceeded at such a slow rate that it was impossible to characterize this reaction.

It is suggested that the enzyme, lipase, is specific for esterified primary hydroxyl groups.

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PURIFICATION AND PROPERTIES OF THE PHOSPHORYLASE-RUPTURING ENZYME

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Two forms of rabbit muscle phosphorylase were described by Cori and Green (1) in 1943: a euglobulin, phosphorylase *a*, having about 65 per cent of its maximal enzymatic activity without the addition of adenosine-5′-phosphate, and a more soluble protein, phosphorylase *b*, which is enzymatically inactive unless adenylic acid is added to the reaction mixture. In 1945, Cori and Cori (2) described an enzyme from rabbit muscle which catalyzes the conversion of phosphorylase *a* to phosphorylase *b*. This enzyme was designated the PR (prosthetic group-removing) enzyme in the belief that adenylic acid, firmly bound to phosphorylase *a*, was split out during conversion to phosphorylase *b*. However, all attempts to demonstrate this as the mechanism of the conversion reaction were unsuccessful. There was no evidence for the appearance of adenylic acid among the products of the conversion, nor for the presence of the nucleotide on phosphorylase *a* (2, 3).

It was recognized in 1953 (4) that the conversion of phosphorylase *a* to phosphorylase *b* was associated with a near or exact halving in molecular weight, *i.e.* that the molecular changes effected by the PR enzyme were of a different nature than removal of a prosthetic group. It has been proposed, however, to retain the name PR (phosphorylase-rupturing) enzyme. The purification of rabbit muscle PR enzyme has been undertaken, and some of its properties have been studied.

EXPERIMENTAL

Estimation of Enzyme Activity—The method of assay described by Cori and Cori (2) has been used to measure PR activity. The conversion reaction is followed by assay for phosphorylase activity under conditions in which only the *a* form is active, *i.e.* in the absence of added adenylic acid. That the disappearance of phosphorylase *a* is due to conversion to phosphorylase *b* rather than to non-specific losses in activity can be established

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by assay also in the presence of adenylate. Under these conditions phosphorylase *b* is fully as active as phosphorylase *a*.

The usual preparation of phosphorylase *a* contains about 5 mg. of protein per ml. of crystal suspension. A fresh dilution (about 1:25) of such a preparation with 0.03 M cysteine, pH 6.6, is used for each PR assay. 1 ml. of the dilute phosphorylase solution is preincubated for 20 minutes in a water bath at 30°. During this period three Klett tubes are prepared, each containing 0.1 ml. of a glucose-1-phosphate (0.064 M)-glycogen (4 per cent) mixture. 1 ml. of PR enzyme, suitably diluted with 0.03 M cysteine, pH 6.6, is added to the phosphorylase solution and incubated at 30°. Immediately after mixing, 0.1 ml. is withdrawn from this reaction mixture and added to 0.1 ml. of prewarmed (30°) glycogen-glucose-1-phosphate mixture for phosphorylase assay. After 5 minutes at 30° the phosphorylase reaction is stopped by the addition of 7 ml. of 0.07 N sulfuric acid. Inorganic phosphate, released during phosphorylase action, is determined in the tube used for this reaction by the method of Fiske and Subbarow (5). Only 0.9 ml. of 5 N sulfuric acid is added to bring the final acidity to 0.5 N.

It has been shown that phosphorylase *a*, in the presence of its substrates, is protected from PR action (2). The initial, or zero time, level of phosphorylase *a* activity is thus measured in the first aliquot. Again at timed intervals (10 and 20 minutes) after mixing the PR enzyme and phosphorylase solutions, 0.1 ml. aliquots are withdrawn and the residual phosphorylase *a* content is measured in the manner described above. The conversion follows, under the conditions of the test, the rate equation for a first order reaction (2). PR activity is expressed in terms of the first order rate constant, k, calculated as $k = 1/t$ (log (units of phosphorylase *a* at $t = 0$)/ (units of phosphorylase *a* at t)). Arbitrarily, $k \times 1000$ equals a PR unit. Protein has been determined by the method of Lowry *et al.* (6).

Method of Purification—A typical protocol of the procedure described here is presented in Table I.

Preparation of Crude Extract. Step 1—Fresh rabbit muscle is passed once through a chilled meat grinder; the ground muscle is then homogenized for 3 minutes in a Waring blendor with 2 volumes of cold, distilled water. The homogenate is centrifuged in a Servall angle centrifuge (8000 × *g*) for 5 to 10 minutes and the supernatant fluid filtered through coarse filter paper.

Acid Precipitation. Step 2—The pH of the extract is usually about 6.0. It is lowered to 5.65 by the addition of M acetate buffer, pH 4.6 (1 to 1.5 ml. of acetate buffer per 100 ml. of extract is usually required).[1] After 5 to 10 minutes in the cold, the flocculent precipitate is centrifuged at 8000 × *g* for about 10 minutes. The precipitate is collected into two tubes and washed once with about 80 ml. of cold, distilled water, then dissolved in a

[1] The pH meter is adjusted to the temperature of the extract.

cold solution of sodium glycerophosphate (0.08 M). The volume of the solution is adjusted to give a protein concentration of about 3 per cent (about 10 ml. per 100 gm. of muscle). The pH of the redissolved precipitate is usually near 7.0 and need not be further adjusted immediately. About an 8-fold enrichment in PR activity is effected by the precipitation. The solution is quite turbid at this stage. It can be frozen without loss of PR activity or taken directly through the next step in the purification.

Alkaline Incubation. Step 3—The pH of the glycerophosphate solution of PR enzyme is raised to 9.4 by the addition of molar sodium carbonate (about 0.25 ml. per 10 ml.) and the solution is incubated in a water bath at

TABLE I

Protocol of Procedure (430 Gm. of Muscle)

Fraction	Total volume	Units per ml.	Total units	Protein	Total protein	Specific activity	Recovery	Purification
	ml.			*mg. per ml.*	*mg.*		*per cent*	
Dissolved acid ppt....	40	28,000	1,120,000	19	760	1,470	54*	8.6* times
After alkaline incubation and removal of denatured protein..	30	38,000	1,140,000	9.4	280	4,042	54	24 times
Supernatant from acetone treatment†....	1	11,000	330,000‡	0.37	11‡	30,000	16	176 "

* In this preparation the PR activity of the crude extract was not determined. The recovery and purification shown here are typical for the acid precipitation step. (A complete protocol of another PR preparation will appear elsewhere (8).)

† Only 1 ml. out of the 30 ml. was processed.

‡ Calculated for the entire volume of 30 ml.

37° for 60 minutes. Upon readjustment to neutrality with 1 M acetate buffer a flocculent precipitate forms which can be discarded after centrifugation without loss of PR activity. About two-thirds of the total protein is removed in this way; hence a purification of about 3 over the acid precipitate is achieved. The enzyme is stable at this stage to freezing at pH 9.4 and at neutrality.

Acetone Treatment. Step 4—The supernatant fluid from the preceding step is divided into portions of 10 ml. each in order to facilitate rapid handling. Each aliquot is cooled to 0°. An equal volume of acetone (at −10°) is added at such a rate that the temperature never rises above 0° and is, at the end of the addition, about −5°. The heavy precipitate of protein is centrifuged immediately. About 2 minutes at 10,000 × *g* are sufficient to pack the precipitate well, while causing little rise in temperature. The well drained precipitate is dispersed in 4 ml. of glycerophos-

phate (0.08 M)-Versene (0.01 M) at pH 9.0 and dialyzed against the same buffer for 1 hour. Much denatured protein remains insoluble during the dialysis and is centrifuged off. The supernatant fluid contains PR enzyme at a high level of purity.

The protein concentration is usually under 0.1 per cent at this stage. Losses are incurred on freezing and thawing. The solution can be lyophilized, however, and taken up in a more concentrated solution without loss in activity. The procedure as described has proved to be very reproducible.

The final product of the purification represents an enrichment in PR activity of about 175-fold over the initial extract. It is calculated that 70 γ of the purified PR enzyme will convert 99 per cent of phosphorylase *a* to phosphorylase *b* in 10 minutes. However, physical studies of PR enzyme as a protein are precluded by the high nucleic acid content of the final material.

Attempts have been made to eliminate the nucleic acid contamination by digestion with ribonuclease and alkaline phosphatase. No loss of PR activity occurred during a digestion of the purified enzyme in which 95 per cent of the non-protein material which absorbs at 260 mμ was made acid-soluble. However, a practical means of recovery of the PR enzyme, free from the added enzymes, has not been found. When the nucleic acid digestion is introduced at earlier stages in the procedure, the usual purification is not achieved in subsequent steps.

It seems that PR activity is associated with the small particles of skeletal muscle. The enzyme remains suspended when the neutralized crude extract from Step 1 is centrifuged at 18,000 $\times$ *g* for 30 minutes. It is separated, however, as a small, glassy precipitate under centrifugation at 100,000 $\times$ *g* for 60 minutes. Acidification in Step 2 promotes aggregation of the particles, facilitating the concentration of PR activity by relatively slow centrifugation. Although PR enzyme is unstable at acid pH, the brief exposure here is well tolerated. The specific activity of acid-precipitated PR enzyme is about equal to that centrifuged from the neutral extract.

The particulate nature of PR enzyme is further manifest in its behavior toward ordinary methods of protein fractionation. PR activity is not recovered in any discrete salt or organic solvent fraction, but, rather, comes out of solution over a wide range of concentrations. The two final steps in the purification involve a selective denaturation of accompanying protein rather than a fractionation.

Effect of Salts and Other Substances on PR Activity—The present work extends some of the earlier observations (2) on the effect of salts on PR activity, but fails to confirm the reported metal (Mn^{++}) activation. The in-

hibition which was seen with phosphate esters and other divalent anions applies as well to monovalent salts. The usual PR activity assay mixture contains 0.03 M NaCl introduced with the neutralized cysteine hydrochloride. Fig. 1 shows the inhibition caused by the addition of NaCl (0.01 and 0.1 M) in excess of this. A linear relationship exists between the log of PR units and the square root of the ionic strength of the assay mixture, con-

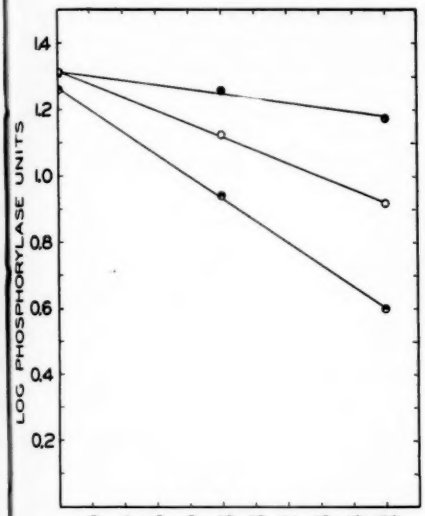

FIG. 1

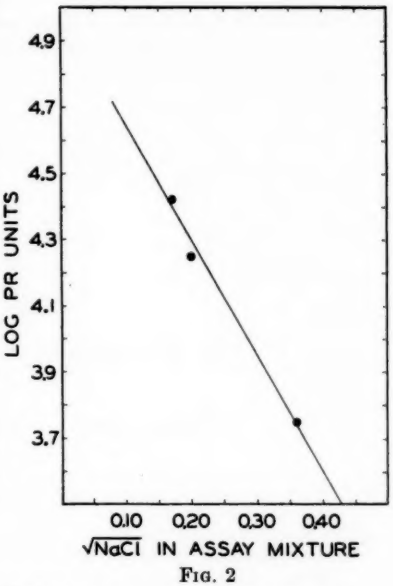

FIG. 2

FIG. 1. The effect of NaCl, 0.1 M (●) and 0.01 M (○), in excess of that (0.03 M, ◓) present in the usual assay mixture.

FIG. 2. The logarithm of PR units is plotted against the square root of the ionic strength of the assay mixture.

firming the ionic nature of the inhibition (Fig. 2). Whereas divalent ions changed the kinetics of the PR reaction from first to zero order (2), NaCl did not affect the kinetics of the reaction. Over and above the general salt effect, NaF is specifically inhibitory to PR activity (7). As is shown in Table II, 50 per cent inhibition is seen at a fluoride concentration near 10^{-3} M.

A marked stimulation of PR activity by cysteine was reported in 1945 (2). Ethylenediaminetetraacetate (crystalline disodium Versenate, pH 6.8, 10^{-3} M) does not substitute for cysteine in this respect. PR enzyme is

only about 20 to 30 per cent as active in a Versene as in a cysteine medium. When Versene (10^{-4} M) is included in the usual assay mixture which contains 0.03 M cysteine, PR activity is inhibited about 30 per cent. Several observations argue against ascribing this effect to the metal-binding capacity of Versene. (1) Overnight dialysis of PR enzyme against 0.08 M

TABLE II

Effect of Fluoride on PR Reaction

NaF was included in the cysteine solution used to dilute the enzyme for assay. In each case the inhibition refers to a control assay mixture containing NaCl at the same molar concentration.

[NaF]	Inhibition
M	*per cent*
10^{-1}	100
10^{-2}	100
10^{-3}	42
10^{-4}	10

TABLE III

Effect of Dialysis and Versene on PR Enzyme

Pretreatment of enzyme	PR activity (units per ml.) assayed in		Inhibition by Versene
	0.03 M cysteine	Same + 10^{-4} M Versene	
			per cent
None	46,500	32,000	31
18 hr. dialysis *vs.* 0.08 M sodium glycerophosphate	45,100		
18 hr. dialysis *vs.* same containing 10^{-4} M Versene	49,600*	36,300	27
Enzyme mixed with Versene (10^{-4} M), then dialyzed 18 hrs. *vs.* glycerophosphate	46,000	30,300	34

* In the PR activity test, Versene is diluted out to a negligible level.

sodium glycerophosphate, pH 9.0, or against this buffer containing 10^{-4} M Versene results in no loss of PR activity. Versene can be mixed with a stock solution of the PR enzyme, then dialyzed out against glycerophosphate without loss of PR activity. Such dialyzed preparations are still inhibited by the presence of Versene in the assay mixture. These experiments are shown in Table III. (2) Addition of another metal-binder, 8-hydroxyquinoline (5×10^{-4} M), to the assay mixture does not depress PR activity. (3) It has not been possible to demonstrate any stimulation of

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PR activity by the divalent metals Mn^{++}, Mg^{++}, or Ca^{++}, nor by boiled juices of PR preparations. In contrast to the Mn^{++} activation noted previously (2), Mn inhibited PR action in all present experiments: 40 per cent inhibition was seen at a Mn^{++} concentration of 5×10^{-4} M and 20 per cent at 5×10^{-5} M. Mn^{++} inhibited whether or not the PR preparation had been dialyzed; the inhibition was noted with PR enzyme prepared by the present method or by the procedure described in 1945. There is as yet no explanation for the discrepant results.

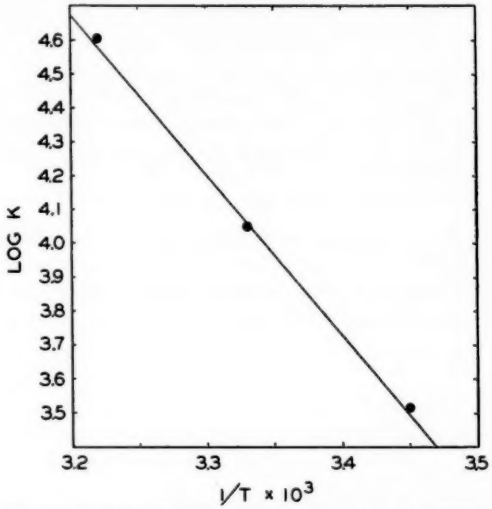

FIG. 3. The logarithm of k, the first order rate constant, $\times$ 1000 is plotted against the reciprocal of absolute temperature. Slope of line, -4720.

The inhibition of PR activity by Versene would seem to reflect some property of the Versene molecule other than that of metal-binding. This property is confined to the non-chelate form of the molecule, since neither Mg Versenate nor Mn Versenate inhibits the PR enzyme.

The temperature coefficient of the PR reaction has been calculated from data obtained at 17°, 27°, and 37°. The Q_{10} was 3.4 and 3.6, respectively, higher values than are found for most enzyme reactions. Fig. 3 presents a plot of log k *versus* $1/T$ for the PR reaction. From the slope of the straight line so obtained (-4720) it is calculated that the energy of activation of the PR-catalyzed conversion of phosphorylase a to phosphorylase b is 21,600 calories.

Mrs. Alice Bronfenbrenner rendered valuable technical assistance.

SUMMARY

The phosphorylase-rupturing enzyme (PR) from rabbit muscle, which is associated with small particles, has been partially purified. The method of purification is based on the following properties of the enzyme: it is precipitated at pH 5.6, it is resistant to incubation at 37° at pH 9.5, and it can be extracted from a precipitate obtained by 50 per cent acetone at −5° at pH 9.5, while most contaminating proteins remain insoluble. An enrichment of about 175-fold over the crude extract has been achieved. Some of the properties of the purified enzyme have been studied.

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THE ACTION OF TRYPSIN ON PHOSPHORYLASE *a*

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Cori and Cori (1) noted in 1945 that crystalline preparations of trypsin simulated the action of the PR (phosphorylase-rupturing) enzyme of muscle on phosphorylase *a*. In an attack which precedes general proteolysis of the molecule, phosphorylase *a* is converted by trypsin to a form enzymatically identical with phosphorylase *b*. The present paper is a study of this reaction, the questions being whether phosphorylases *b* formed by trypsin and by the PR enzyme are, in fact, identical.

Materials and Methods

We are indebted to M. Laskowski for a generous supply of trypsin (recrystallized three times) and pancreatic trypsin inhibitor (recrystallized five times) used for these experiments. Phosphorylase *a* was prepared by the method of Green and Cori (2); phosphorylase *b* was made from phosphorylase *a*, either by the action of crystalline trypsin or by purified PR enzyme, as specified in the text.

The procedure for following the conversion of phosphorylase *a* to phosphorylase *b*, catalyzed by trypsin, is the same as that used when the conversion is catalyzed by PR enzyme (3).

EXPERIMENTAL

Effect of pH

The trypsin-catalyzed conversion of phosphorylase *a* to phosphorylase *b* was carried out in imidazole buffers of five different pH values ranging from 6.1 to 7.4. The units of phosphorylase *a* activity were measured at zero time and at intervals (2 and 4 minutes) after addition of trypsin. The short reaction periods were chosen in order to minimize any effect of the instability of trypsin at higher pH values. Trypsin activity was maintained in the second time period at all but the highest pH. At pH 7.4 the rate constant was about 22 per cent lower in the second period. Fig. 1 presents the pH-activity curve for the reaction which proceeds optimally in imidazole buffer at pH 6.8. This is about 1 pH unit below the value

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reported for most trypsin reactions. The rate of the conversion falls off sharply on the alkaline side of pH 6.8.

Ultracentrifugal Studies

Solutions of phosphorylase *a* were incubated with trypsin to effect partial conversion to phosphorylase *b*. The action of trypsin was stopped at definite intervals by the addition of pancreatic trypsin inhibitor in excess. The reaction mixtures were examined in the ultracentrifuge and by enzy-

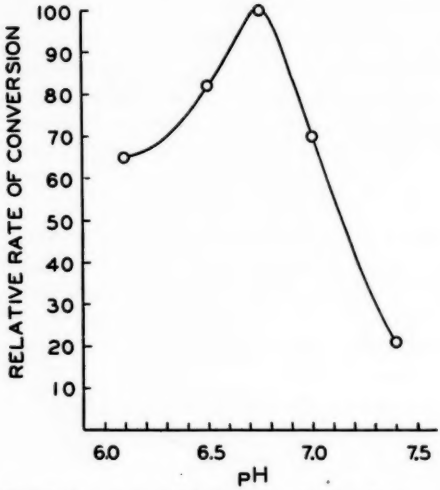

FIG. 1. The pH-activity curve of the trypsin-catalyzed conversion of phosphorylase *a* to phosphorylase *b*. Each reaction mixture contained 0.010 mg. of trypsin and 0.160 mg. of phosphorylase per ml. of imidazole buffer (0.01 M).

matic assay. While solutions of phosphorylase *a* alone are ultracentrifugally homogeneous, solutions incubated with trypsin contained a new protein component.[1] The second component arose at the expense of phosphorylase *a* and sedimented as a single molecular species.

Table I presents the data by which the second protein component is identified as phosphorylase *b*. For each experiment 1 ml. of phosphorylase solution (8 mg. of protein) was dialyzed for several hours against imidazole buffer (0.01 M, pH 6.8). Trypsin was added, in the amounts indicated, and the mixtures were incubated at 30°. Trypsin inhibitor (38 γ) was added to stop further action of the enzyme and an aliquot was removed for enzymatic assay. Concurrently, as the remainder was ultracentrifuged

[1] The amounts of trypsin added were too small to be seen in the ultracentrifuge.

(Spinco ultracentrifuge, model E) at 250,000 × g, the aliquot was assayed for phosphorylase activity in the presence and in the absence of added adenylic acid.[2] From the ratio of these two activities, before and after incubation, the per cent of phosphorylase b in the mixture can be calculated. Enlarged tracings were made of the sedimentation diagrams and the relative areas of the two protein components were measured planimetrically. It will be seen in Table I that the relative area of the second protein component is at all times in good agreement with the proportion of phosphorylase b in the mixture as indicated by enzymatic assay.

TABLE I

Comparison of Ultracentrifugal and Enzymatic Analyses of Incubation Mixtures of Phosphorylase a and Trypsin

Phosphorylase a, at a concentration of 8 mg. per ml. in imidazole buffer, pH 6.8, was incubated with trypsin as indicated. Pancreatic trypsin inhibitor was added to stop the reaction and aliquots were analyzed enzymatically and ultracentrifugally as described in the text.

Trypsin added	Time incubated	Phosphorylase b by enzymatic assays	2nd component by area analyses
mg. per ml.	*min.*	*per cent*	*per cent*
0.005	7	18	17
0.005	30*	28	28
0.005	15	41	47
0.005	30	56	53
0.010	30	76	76

* The pH of this reaction mixture was 7.4.

In Fig. 2 the sedimentation constant of phosphorylase b, formed by trypsin or by PR enzyme, has been plotted against protein concentration. Both sets of values fall on a straight line, extrapolating to 8.2 S at infinite dilution. The sedimentation constant of phosphorylase a at zero protein concentration is 13.2 S. Addition of AMP during centrifugation did not affect the sedimentation constants of phosphorylases a or b.

Diffusion Constant

An attempt has been made to measure the diffusion constant of trypsin-made phosphorylase b. It is difficult to effect complete conversion of large amounts of phosphorylase a to phosphorylase b by trypsin without incurring some loss through general proteolysis. For this experiment it seemed preferable to retain full enzymatic activity even at the expense of complete conversion. The diffusion experiment was carried out with a mixture of

[2] Adenosine-5′-phosphate, abbreviated elsewhere in the text as AMP.

known composition, and the observed constant was treated as a weight average of the diffusion constants of phosphorylases *a* and *b*.

32.3 mg. of phosphorylase *a* were first dialyzed against imidazole buffer (0.01 M, pH 6.8) and then incubated with 0.23 mg. of trypsin. After addition of trypsin inhibitor the mixture, containing, according to enzymatic analysis, 75 per cent phosphorylase *b* and 25 per cent phosphorylase *a*, was dialyzed to equilibrium against potassium phosphate-potassium chloride buffer of ionic strength 0.23 and pH 7.1. Free diffusion against the buffer was allowed to proceed in a Tiselius electrophoresis cell at 2° for 53 hours. An unexplained disturbance was present in one limb of the cell from the outset of the experiment. Schlieren scanning photographs of the boundary in the remaining limb presented diffusion curves which were symmetrical

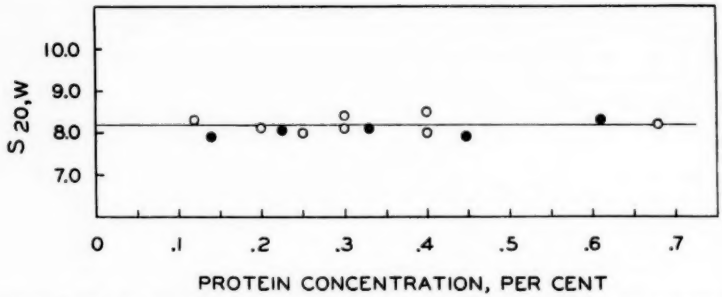

FIG. 2. The sedimentation constants of phosphorylase *b* made by trypsin (●) or by the PR enzyme (○) plotted as a function of protein concentration.

over the 53 hour period. Enlarged tracings were made of the diffusion curves and u, one-half the distance between the inflection points, was measured for each curve. The diffusion constant was calculated from the equation $D = u^2/2t$ and corrected to obtain the value at 20° in water. The observed constant (2.96) was resolved as

$$D_{obs.} = \frac{(D_a \times \text{weight \% phosphorylase } a) + (D_b \times \text{weight \% phosphorylase } b)}{\text{weight \% phosphorylase } a + \text{weight \% phosphorylase } b}$$

$D_{20,w}$ for phosphorylase *a* is 2.6×10^{-7} sq. cm. per second (4). When this value and the respective weight percentages of each component were substituted into the equation, the diffusion constant of trypsin-made phosphorylase *b* was calculated to be 3.1×10^{-7} sq. cm. per second.

Green (5) has measured the diffusion constant of phosphorylase *b* made by PR action on phosphorylase *a*. She reports the figure 3.3×10^{-7} sq. cm. per second as $D_{20,w}$. The molecular constants of phosphorylase *a* and of the two kinds of phosphorylase *b* are summarized in Table II. It will be

seen that the molecular weights of both forms of phosphorylase *b* are about one-half that of phosphorylase *a*. Whether the 6 per cent difference seen between the molecular weights of trypsin and PR-made phosphorylase *b* is real or apparent cannot be decided by these methods. The frictional ratios, f/f_0, of the three forms of phosphorylase do not appear to differ significantly from each other.

Electrophoretic Properties

The electrophoretic behaviors of the two forms of phosphorylase *b* have been compared. Solutions of phosphorylase *a* were incubated with trypsin and with PR enzyme, respectively. In each case, when conversion to

TABLE II

Molecular Constants of Phosphorylase a and Two Species of Phosphorylase b

The diffusion constant of PR-made phosphorylase *b* was determined by Green (5). The partial specific volumes ($\bar{V}_{20}$) of phosphorylase *a* and of PR-made phosphorylase *b* have been determined (4). The same value has been assumed for $\bar{V}_{20}$ of trypsin-made phosphorylase *b*.

Constant	Phosphorylase *a*	Phosphorylase *b* (PR)	Phosphorylase *b* (trypsin)
$S_{20,w} \times 10^{13}$	13.2	8.2	8.2
$D_{20,w} \times 10^{7}$	2.6	3.3	3.1
$\bar{V}_{20}$	0.750	0.750	0.750
Mol. wt.	495,000	242,000	257,000
$\frac{f}{f_0}$	1.55	1.55	1.65

phosphorylase *b* had occurred and trypsin action had been stopped by the inhibitor, the entire reaction mixture was dialyzed against potassium phosphate buffer of ionic strength 0.1, pH 7.8. Electrophoreses were conducted at 2° in a Tiselius apparatus.

35 mg. of phosphorylase *a* were incubated with 0.14 mg. of trypsin to effect 87 per cent conversion in 15 minutes with 90 per cent retention of enzymatic activity. The phosphorylase *b* formed in this reaction migrated as a single protein across the distance of the cell. Similarly, phosphorylase *b* made by PR action (320 γ of PR per 22 mg. of phosphorylase *a*) was electrophoretically homogeneous. However, phosphorylase *b* made by trypsin carries a greater net negative charge under these conditions than does PR-made phosphorylase *b*. The electrophoretic mobilities of the two proteins are shown in Table III. The difference was confirmed in another experiment in which a mixture of known proportions of the two forms of phosphorylase *b* was examined. In this experiment the proteins separated

during electrophoresis, the trypsin product moving more rapidly toward the positive electrode.

As with the PR enzyme (6), the trypsin-catalyzed conversion of phosphorylase *a* to phosphorylase *b* is inhibited by AMP. While 20 γ of trypsin effected 71 per cent conversion of 6 mg. of phosphorylase *a* to phosphorylase *b* in 12 minutes in the absence of AMP, no conversion occurred under the same conditions when AMP (10^{-3} M) was present in the reaction mixture. Neither NaF nor NaCl (0.1 M) has any effect on the trypsin reaction, whereas PR enzyme is inhibited by salts generally and by fluoride specifically (3, 6). Conversely, trypsin action is effectively halted or prevented by specific trypsin inhibitors (soy bean and pancreatic) which were found to have no effect on the PR enzyme. The Michaelis constant (K_m) of the two forms of phosphorylase *b* for AMP was determined at 30°. The activity of each enzyme was half maximal at a concentration of 3×10^{-5} M AMP. The value of 5×10^{-5} M was reported by Cori, Cori, and Green (7) for phosphorylase *b* made by PR action. The deoxyribose analogue of AMP was tested with PR-made phosphorylase *b*; it did not activate.

TABLE III

Electrophoretic Mobilities of Phosphorylases b in Potassium Phosphate Buffers of Ionic Strength 0.1, pH 7.8

Phosphorylase *b*	u, cm.² per sec. per volt
PR-made	-3.7×10^{-5}
Trypsin-made	-4.7×10^{-5}
Mixture, 65% PR-made	-3.9×10^{-5}
" 35% trypsin-made	-4.5×10^{-5}

DISCUSSION

The experiments which have been described establish that the trypsin-catalyzed conversion of phosphorylase *a* to phosphorylase *b* is of the same general type as the PR reaction. In both reactions there is an approximate halving in the molecular weight of phosphorylase *a*. The phosphorylase *b* molecules formed by trypsin and by PR enzyme are indistinguishable in the ultracentrifuge, but are not identical since they can be separated electrophoretically.

Trypsin-made phosphorylase *b* carries a greater net negative charge in phosphate buffers of ionic strength 0.1, pH 7.8, than does PR-made phosphorylase *b*. The difference in charge cannot be readily explained. Several possibilities suggest themselves. Trypsin might split out a peptide fragment at the site of cleavage or it might split off amide groups. Velick

and Wicks (8) have suggested that phosphorylase binds anions at pH values below 10. If this is so, the electrophoretic behavior of the two forms of phosphorylase *b* may be due to a difference in phosphate binding.

Krebs (9) has found another difference in the behavior of the two species of phosphorylase *b*. While salmine increased the affinity for AMP of phosphorylase *b* made by PR action, this was not the case for trypsin-made phosphorylase *b*. Whatever the exact nature of the two forms of phosphorylase *b* they have similar or identical molecular weights, the same specific enzymatic activity, and an equal affinity for AMP in the absence of salmine.

No conclusions can be drawn from these properties of the two forms of phosphorylase *b* as to the mode or site of action of trypsin and of PR enzyme. End-group determination of phosphorylase *a* and of the two forms of phosphorylase *b* are in progress. It is hoped that they will establish whether the attack of trypsin on phosphorylase *a* is consistent with its known specificity requirements. They may also help to elucidate the mechanism of PR action.

SUMMARY

The trypsin-catalyzed conversion of phosphorylase *a* to phosphorylase *b* has been studied. The reaction is associated with an approximate halving in the molecular weight of phosphorylase *a*. Phosphorylase *b* made by trypsin is ultracentrifugally indistinguishable from phosphorylase *b* made by the PR enzyme, but is electrophoretically distinct.

I wish to thank Dr. Gerty T. Cori and Dr. Carl F. Cori for their guidance in this work, and Miss Carmelita Lowry for generous assistance in making the physical measurements.

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INHIBITION OF THE PHOSPHORYLASE-RUPTURING ENZYME BY SOME TRYPSIN SUBSTRATES

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The analogous activities of trypsin and the PR (phosphorylase-rupturing) enzyme (1) in converting phosphorylase *a* to phosphorylase *b* suggest a possible mechanism of action for the PR enzyme. Although phosphorylase *b* made by trypsin is not identical with that made by the PR enzyme (2), the differences which have been seen do not preclude a similar mechanism of action for the two enzymes. Presumably this would be a proteolytic or esteratic attack on phosphorylase *a*.

Cori and Cori (1) examined partially purified preparations of PR enzyme for proteolytic activity in 1945. No increase in non-protein nitrogen was noted when PR enzyme was incubated with dialyzed rabbit serum, with muscle protein fractions, or with phosphorylase *a*. It now seems, however, from its action on phosphorylase *a* (3), that PR enzyme might be an endoproteinase capable of splitting only one or a few internal bonds of the protein.

The question of proteolytic action by the PR enzyme has therefore been reexamined with different methods, the more highly purified preparations of PR enzyme now available being used. Experiments were designed to test further for trypsin-like activity by the PR enzyme. Although PR enzyme, under the conditions employed, did not appear to act as an endoproteinase on other muscle proteins nor split any of the trypsin substrates tested, it was inhibited in its action on phosphorylase *a* by the ethyl esters of L-arginine and L-lysine. The kinetics of the inhibition appeared to be those of competition with phosphorylase *a* for the PR enzyme.

EXPERIMENTAL

Muscle Proteins As Substrates

Two crystalline proteins from rabbit muscle were tested as substrates for PR action. In view of the possibility that only a few fragments of relatively high molecular weight might result, the incubation mixtures were examined in the ultracentrifuge. The desired level of PR activity could be achieved by amounts of protein too small to interfere optically.

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Myosin, like phosphorylase *a*, is attacked by trypsin in a specific and differential manner (4). This protein was tested as a substrate for PR action. Twice recrystallized, ultracentrifugally homogeneous myosin, prepared by the method of Mihályi (5), was incubated, at a concentration of 4 mg. per ml., with 1300 units per ml. of PR enzyme (for definition of PR units see Keller and Cori (6)). The solution was 0.2 M in KCl, 0.01 M in cysteine, and 7×10^{-4} M with respect to sodium glycerophosphate at pH 6.8. After 20 hours of incubation at 30° the mixture was examined in the ultracentrifuge (Spinco, model E) at 250,000 × *g*. A single component, apparently identical with untreated myosin, was seen.

An enzyme from the glycolytic cycle was also tested as a substrate for PR activity. Aldolase recrystallized three times, prepared according to Taylor *et al.* (7), was incubated at a concentration of 2.6 mg. per ml. with 600 units of PR enzyme. The mixture contained 0.01 M KCl and 0.015 M cysteine at pH 6.7. After 6 hours of incubation at 30° the mixture was ultracentrifugally indistinguishable from untreated aldolase.

In each case the concentration of PR enzyme was several times that necessary for complete conversion of similar amounts of phosphorylase *a* to phosphorylase *b* in 1 hour.

Effect of PR Enzyme on Esters

N-Acylated and unacylated esters of the basic amino acids are known to be substrates for trypsin (8). Their enzymatic hydrolysis is usually followed by titration of the carboxyl groups released during the reaction.

Three compounds of this type were examined as substrates for PR enzyme: benzoyl-L-arginine ethyl ester,[1] L-lysine ethyl ester, synthesized as described by Werbin and Palm (9), and L-arginine ethyl ester which was synthesized by the same procedure used for the lysine ester. The reactions were followed titrimetrically. PR enzyme was added to a solution of the substrate at a preselected pH and the amount of standard NaOH required to maintain that pH was measured at regular intervals. The mixture was magnetically stirred, the temperature was maintained at 37°, and the pH was measured with grounded and shielded micro electrodes connected to a Beckman model G pH meter. The standard NaOH was added beneath the surface of the reaction mixture from a Machlett micro autoburette fitted with a 3 inch, No. 20 hypodermic needle. The method was found to be sensitive to less than 1 per cent hydrolysis of the substrates.

None of the esters was hydrolyzed to a measurable degree by 3000 to 5000 units of PR enzyme. All were hydrolyzed by trypsin at enzyme con-

[1] Kindly supplied by Dr. Hans Neurath.

centrations equivalent, in terms of the conversion of phosphorylase *a* to *b*, to about 300 PR enzyme units per ml.

Inhibition Studies

An alternative method of elucidating the mechanism of enzyme action is through the use of inhibitors. The three esters studied above and three other compounds were examined for their effect on the PR-catalyzed conversion of phosphorylase *a* to *b*.

The activity was measured as described (6), except that a single time interval of 10 minutes was chosen. The substrate concentration was varied over a 10-fold range, *i.e.*, from 185 to 2070 units of phosphorylase *a* per ml. of reaction mixture. These concentrations are all well below the level of phosphorylase *a* which saturates the PR enzyme. The first order velocity constant, k', was calculated for each experiment. The initial substrate concentration [*S*] was multiplied by k' to obtain the initial rate of the reaction, v. The reciprocals of initial rate and of initial substrate concentration were plotted against each other, according to Lineweaver and Burk (10).

The esters were dissolved in the cysteine solution used to dilute PR enzyme. The enzyme was preincubated with the ester (10^{-2} M) for 2 minutes at 30° before addition to phosphorylase *a*. Controls, without ester, were treated identically in terms of dilution and preincubation. The final concentration of ester in the assay mixture was 5×10^{-3} M, except in the case of L-arginine ethyl ester when it was 6×10^{-3} M.

The ethyl esters of L-arginine and L-lysine inhibited PR action on phosphorylase *a*. The kinetics of the inhibition are shown in Fig. 1. While the slopes of the $1/v$:$1/[S]$ plots differ significantly, the intercepts do not. By these criteria the inhibition of PR enzyme by L-arginine ethyl ester and L-lysine ethyl ester is of the competitive type. L-Leucine ethyl ester (11) did not inhibit PR action, nor did benzoyl-L-arginine ethyl ester. L-Arginine inhibited only 6 per cent, the effect being independent of phosphorylase *a* concentration. While L-lysine inhibited 13 per cent, L-lysine ethyl ester inhibited more than 50 per cent under the same conditions. The small inhibitions by arginine and lysine are within the range of salt effects on the PR enzyme (6).

By applying the method of least squares to the data of Fig. 1 the best straight line was drawn. K_s for PR enzyme was calculated to be 3000 units of phosphorylase *a* per ml. This corresponds to 1 mg. of phosphorylase, recrystallized to constant specific activity (12). By taking 495,000 as the molecular weight of the protein (3), K_s can be expressed as 2×10^{-6} M phosphorylase *a*. K_i for L-arginine ethyl ester was 3.3×10^{-3} M; for L-lysine ethyl ester it was 6.6×10^{-3} M. The affinity of PR enzyme for

these compounds thus averages about 1/2500 that for phosphorylase *a*. $V_{max.}$ is 1.2×10^{-5} μmole per ml. of phosphorylase *a* converted to phosphorylase *b* in 1 minute at a concentration of 1 PR unit per ml.

The relative affinities of PR enzyme for phosphorylase *a* and for L-arginine ethyl ester were considered in another attempt to show hydrolysis of L-arginine ethyl ester directly. 76,000 units of highly purified PR enzyme

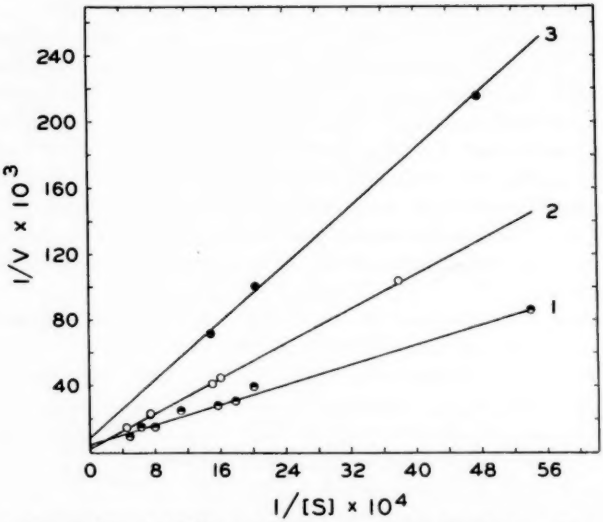

FIG. 1. Competitive inhibition of PR action on phosphorylase *a*. Curve 1, phosphorylase *a* alone; Curve 2, plus 5×10^{-3} M L-lysine ethyl ester; Curve 3, plus 6×10^{-3} M L-arginine ethyl ester. The initial rate, *v*, was determined as described in the text and is expressed as units of phosphorylase *a* converted to phosphorylase *b* per ml. per minute. *S*, the initial substrate concentration, is expressed in units of phosphorylase *a* per ml. The PR concentration was 25 units per ml.

(30,000 units per mg. of protein) in 2.5 ml. of cysteine (0.015 M, pH 6.8) were added to a neutralized solution of L-arginine ethyl ester. The final concentration of the ester was 0.065 M, or about 20 times the dissociation constant for this ester and PR enzyme; the concentration of PR enzyme was 25,000 units per ml. or 1000 times that used in conversion reactions. The reaction was followed titrimetrically as described above. No evidence of hydrolysis was seen in 1 hour's incubation at 38°.

If PR enzyme had catalyzed the hydrolysis of L-arginine ethyl ester at the same maximal rate as was found for the conversion of phosphorylase *a* to *b* (1.2×10^{-5} μmole per ml. per minute per unit of PR activity), 0.3

μmole of ester would have been hydrolyzed per minute by 25,000 units of PR enzyme in 1 ml. Less than 0.01 μmole per ml. per minute actually was hydrolyzed. Thus if PR enzyme has any action on L-arginine ethyl ester, it proceeds at less than 1/30 the rate of the conversion reaction.

Trypsin, on the other hand, at a concentration of 0.02 mg. per ml. (280 units in terms of conversion of phosphorylase *a* to *b*) effected 40 per cent hydrolysis of L-arginine ethyl ester in 1 hour. In Fig. 2 the activities of PR enzyme and trypsin toward L-arginine ethyl ester are compared.

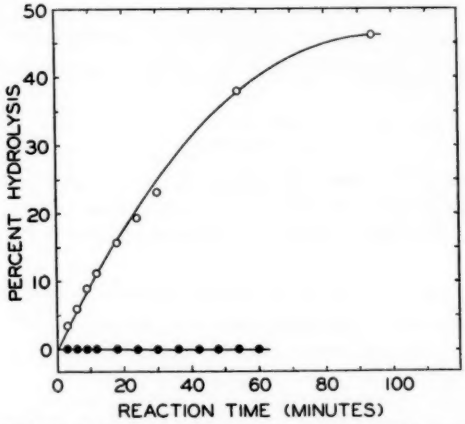

FIG. 2. Comparison of action of PR enzyme and trypsin on L-arginine ethyl ester. L-Arginine ethyl ester was incubated with 25,000 units per ml. of PR enzyme (●) and with trypsin (○) equivalent to 280 PR units per ml. Both reactions were carried out at 37° and pH 6.8.

DISCUSSION

The PR enzyme has been tested as a catalyst in several reactions known to be mediated by trypsin. Based on the specific nature of the action of PR enzyme on phosphorylase *a*, it was not expected that any extensive similarity between the two enzymes would be observed. It was felt, however, that trypsin and PR enzyme might have in common some specific initial attack on other proteins, the nature of which would help to characterize the PR enzyme. No such common action has been found. Neither myosin nor aldolase was observed to be affected by PR action.[2] PR enzyme had no measurable action on amino acid esters which are readily hydrolyzed by trypsin (8).

[2] Chymotrypsinogen was not converted to chymotrypsin by PR enzyme (G. T. Cori, unpublished).

However, support for the idea that there are similarities between trypsin and PR action comes from the competitive inhibition of PR activity by some of the trypsin substrates. There are several indications of specificity of this inhibition. Arginine and lysine fail to inhibit PR activity competitively, while the corresponding ethyl esters do, indicating that the carboxyl group must be covered. The inactivity of leucine ethyl ester as an inhibitor emphasizes the affinity of the enzyme for a basic side chain, a characteristic of trypsin (13). That benzoyl-L-arginine ethyl ester does not inhibit PR activity under the conditions employed may be a reflection of a lowered affinity of PR enzyme for this compound as compared with arginine ethyl ester. This is paralleled by a lowered activity of trypsin towards benzoyl-L-arginine ethyl ester when compared with L-arginine ethyl ester.[3]

SUMMARY

The PR enzyme has been tested for action on several typical trypsin substrates. No hydrolytic action was observed on crystalline myosin or aldolase, nor on the ethyl esters of L-arginine, benzoyl-L-arginine, or L-lysine.

The ethyl esters of arginine and lysine competitively inhibited the PR-catalyzed conversion of phosphorylase *a* to phosphorylase *b*. K_s of PR enzyme is 2×10^{-6} M phosphorylase *a*; K_i is 3.3×10^{-3} M for L-arginine ethyl ester and 6.6×10^{-3} M for L-lysine ethyl ester.

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[3] M. Fried, unpublished experiments.

THE BIOSYNTHESIS OF SUCROSE*

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Hassid, Doudoroff, and Putman (1–3) discovered an enzyme in certain bacteria (*Pseudomonas saccharophila*) which catalyzes the following reaction:

Glucose-1-phosphate + fructose ⇄ sucrose + inorganic phosphate

The enzyme, which was named sucrose phosphorylase, has not been found in plant tissues (4), and thus the mechanism of sucrose synthesis remains obscure. Evidence obtained from tracer experiments led Buchanan *et al.* (5, 6) to assume that in plants sucrose phosphate is formed from UDPG[1] and fructose-1-phosphate. This hypothesis stimulated work which led to the discovery of an enzyme which catalyzes sucrose synthesis according to the following reaction:

$$\text{UDPG} + \text{fructose} \rightleftarrows \text{sucrose} + \text{UDP} \qquad (1)$$

A brief note (7) reported the preparation of this enzyme from wheat germ and its presence in some other plant materials. Furthermore, the validity of Reaction 1 was proved by the equivalence between the disappearance of UDPG and the formation of UDP and sucrose. A more detailed study is reported in this paper.

Methods

Analytical—Sucrose was estimated by the resorcinol method of Roe (8). The volumes taken were reduced to one-fourth, and the color was measured at 490 mμ. In order to avoid the interference of fructose, the samples were heated 10 minutes at 100° after adding sufficient sodium hydroxide to make the concentration 0.2 N. While 2 μmoles of fructose were found to give no color with resorcinol after this treatment, sucrose remained unaffected even when the concentration of alkali was 0.5 N. High concentrations of monosaccharides yield some color, even after alkaline treatment, but this can be corrected by the use of suitable blanks.

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[1] The following abbreviations are used: UDP for uridine diphosphate, UDPG for uridine diphosphate glucose, and Tris for tris(hydroxymethyl)aminomethane.

The method of Kunitz and McDonald (9) was followed for protein estimation.

Substrates—UDPG and UDP were prepared as described previously (10). The sugars were commercial samples.

Estimation of Enzyme—The following components were mixed: 0.5 μmole of UDPG, 2 μmoles of fructose, 0.01 ml. of 2 M Tris buffer of pH 7.2, and variable amounts of enzyme; total volume, 0.15 ml. After 30 minutes at 37°, water was added to 0.5 ml., followed by 0.02 ml. of 5 N sodium hydroxide. After careful mixing, the tubes were heated 10 minutes at 100° and sucrose was estimated. An equal sample, in which UDPG was added after the incubation, and sucrose standards (0.1 to 0.2 μmole) were run at the same time.

TABLE I

Purification of Enzyme

Fraction	Volume	Units*	Purity†
	ml.		
I. Crude extract	70		~0.05
II. 1st ammonium sulfate	35	672	0.24
III. Manganous chloride	40	132	0.66
IV. 2nd ammonium sulfate	20	108	1.16
V. Alumina, 1st supernatant	20	104	1.80
VI. " 2nd "	20	72	2.40

* 1 unit is defined as the amount of enzyme catalyzing the formation of 1 μmole of sucrose in 30 minutes under the conditions described in the text.

† Expressed in units per mg. of protein.

Plant Material—Beet, sweet sorghum, or pea seeds were allowed to germinate 4 to 5 days at 30° under light on wetted cotton. The shoots were then ground, and the solids were removed by filtration through muslin. The liquid was then treated with ammonium sulfate, and the fraction obtained between 0.25 and 0.60 saturation was redissolved and dialyzed overnight in the refrigerator against distilled water. The protein content of the extracts was found to decrease with the age of the plantules.

Sugar cane shoots (2 to 3 cm. long) and roots were obtained from stem cuttings which had been kept in the laboratory on wetted cotton at 30°. The extracts were prepared as described above.

Purification of Wheat Germ Enzyme—30 gm. of commercial wheat germ and 100 ml. of 0.05 M phosphate buffer of pH 7.2 were mixed in a blender. The suspension was centrifuged 15 minutes at 16,000 r.p.m., and the supernatant fluid was dialyzed with constant stirring at 5° during 4 to 5 hours. The liquid was centrifuged again as before (Fraction I, crude extract (Table I)).

The supernatant solution was made 0.5 saturated with solid ammonium sulfate, and the precipitate obtained by centrifugation was dissolved in half the volume of Fraction I of water and dialyzed overnight at 5° against distilled water (Fraction II). 0.1 volume of 1 M manganous chloride was added, and the suspension was stirred during 30 minutes at 0°. After centrifugation the supernatant fluid (Fraction III) was made 0.3 saturated with ammonium sulfate, and the precipitate was discarded. Ammonium sulfate to 0.5 saturation was added, and the precipitate was dissolved in

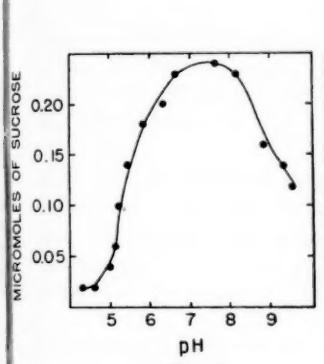

FIG. 1

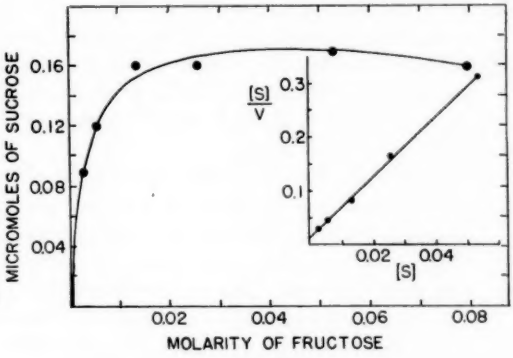

FIG. 2

FIG. 1. pH optimum curve. System as described in the text with Tris or acetate buffer at 0.15 M final concentration. Incubated 15 minutes at 37°. The pH was determined on aliquots with a glass electrode.

FIG. 2. Influence of fructose concentration. System as described in the text. The amount of purified enzyme corresponded to 0.18 mg. of protein. Fructose concentration as indicated. Section at right, a Lineweaver-Burk (22) plot. $K_m = 2.3 \times 10^{-3}$.

half the volume of Fraction III of water and dialyzed during 1 to 2 hours in the cold with constant stirring (Fraction IV).

Alumina (C_γ) (usually 0.1 volume of a suspension containing 50 mg. of dry weight per ml.) was added to the liquid, and the precipitate was discarded. To the supernatant fluid more alumina was added (0.2 volume), and the precipitate was again discarded. The supernatant solution (Fraction VI) was the best preparation obtained (Table I).

Results

Properties of Enzyme—In crude extracts nearly all the activity could be recovered in the precipitate from adding acetic acid to pH 5. However, no appreciable purification was obtained by this procedure.

Heating 10 minutes to 60° led to nearly complete destruction of the

wheat germ enzyme. Considerable destruction was found to take place on precipitation with acetone, even at low temperature. The enzyme could be stored for months in the frozen state without much loss of activity.

Inhibitors—The following substances did not affect the activity: arsenate, arsenite, fluoride, iodoacetate, citrate, or pyrophosphate at 0.01 M concentration; calcium, barium, or magnesium ions at 0.05 M concentration; phlorizin, 8-hydroxyquinoline, or ethylenediaminetetraacetate at 0.2 saturation.

pH Optimum—As shown in Fig. 1, the highest activity was obtained at pH 7.2 with 0.15 M Tris buffer.

Substrate Concentration—The result of an experiment with different concentrations of fructose is shown in Fig. 2. If the amount of sucrose formed is taken as a measure of rate of reaction, calculation of the Michaelis constant was 2.3×10^{-3}. A 2-fold increase of UDPG concentration did not affect the rate of reaction.

Specificity—No substance reacting like sucrose was found to be formed when UDPG was replaced by glucose-1-phosphate, fructose-1- or 6-phosphate, UDP-acetylglucosamine (11), or guanosine diphosphate mannose (12), or when fructose was replaced by sorbose or by fructose-6-phosphate. However, the latter was true for only a few of the preparations obtained. This point will be dealt with in the following paper.

In other tests, the disappearance of UDPG was measured by a method based on its activity as cogalactowaldenase (10). When fructose was added to the wheat germ enzyme and UDPG, the disappearance of the latter was increased. No increased disappearance was observed if fructose was replaced by D-glucose, D-galactose, D-mannose, D- or L-arabinose, D-ribose, or inositol.

Reaction Product—The isolation of crystalline sucrose was not attempted, since it would have required considerable amounts of UDPG. However, the tests which have been carried out make it reasonably certain that the product is sucrose.

Paper chromatography of the reaction products revealed the presence of a substance which gave the R_F value of sucrose (Table II). This substance was absent in controls in which the fructose or the UDPG was added at the end of the incubation period. The substance behaved like sucrose when the papers were developed with the alkaline silver (13), resorcinol (14), or benzidine-trichloroacetic acid reagents (15).

In other experiments, the reaction product was isolated by paper chromatography. The substance was found to have no reducing power and to behave like sucrose during paper chromatography. After mild acid hydrolysis (5 minutes, pH 2, 100°) or after treatment with yeast invertase, glucose and fructose could be detected chromatographically.

With the solvents used for paper chromatography (ethyl acetate-pyri-

dine-water (10:5:6) (16) and butanol-acetic acid-water (17)) and with the reagents used for revealing the spots, sucrose can be easily distinguished from maltose, trehalose, lactose, and raffinose.

TABLE II

Paper Chromatography of Reaction Product

	R_{xylose}			
	Pyridine-ethyl acetate		Butanol-acetic acid	
Complete system	0.82	0.94	0.32	0.80
No UDPG*		0.93		0.82
" fructose*		0.93		0.83
Sucrose		0.82		0.32
Glucose		0.87		0.58
Fructose		0.93		0.82
Maltose		0.72		0.20
Trehalose		0.75		

* Added at the end of the incubation period.

TABLE III

Acid Hydrolysis of Reaction Product

The samples (0.18 μmole) were heated at 100° in 1 ml. of 0.1 M glycine-hydrogen chloride buffer of pH 2.25. Samples of sucrose were run at the same time under identical conditions. The reducing power was measured with ferricyanide (23). Results in per cent hydrolysis.

	Time		
	2 min.	4 min.	6 min.
Sucrose	36	70	82
Reaction product	32	73	85

The rate of acid hydrolysis of a known sample of sucrose was compared under identical conditions with a sample of the reaction product; both substances hydrolyzed at the same rate (Table III).

Reversibility—In order to detect the reversibility, the back-reaction was investigated by starting with 5 μmoles of sucrose, 2 μmoles of UDP, and enzyme. The mixture was deproteinized with trichloroacetic acid, and, after extracting the latter with ether, the samples were chromatographed on paper with an ethanol-ammonium acetate solvent of pH 7.5 (18) containing 0.01 M ethylenediaminetetraacetate. A spot having the same mobility as UDPG was visible under ultraviolet light. After extraction of the substance from the paper, UDPG was estimated by its cogalactowaldenase activity and absorption at 260 mμ. The amount obtained was

about 0.05 μmole. Control samples in which one of the reactants was omitted during the incubation gave no UDPG spot or cogalactowaldenase activity.

Many attempts to obtain a precise figure for the equilibrium constant were carried out by starting with known mixtures of reactants and products. The reducing power or the sucrose content of the samples was measured before and after enzyme action. A small correction had to be applied, owing to the liberation of reducing power from sucrose, which occurred even with the most purified enzyme preparations. Many experiments were carried out in this manner, but the results were not consistent and the value for K = (sucrose × UDP)/(UDPG × fructose) varied from 2 to 8 at 37° and pH 7.4 in different experiments.

Distribution—The detection of the enzyme in some plant tissues is difficult, owing to the presence of sucrase. However, it has been possible to obtain extracts from many sources which catalyze the formation of sucrose, and in general seeds were the best materials for the preparation of the enzyme. Quantitative measurements were carried out with some extracts, and the results were as follows (in micromoles of sucrose formed in 30 minutes per mg. of protein): beet shoots 0.6 to 1.0, sweet sorghum shoots 0.7 to 4.0, sugar cane shoots 0.25, sugar cane roots 2.4 to 3.0, pea shoots 0.35. Qualitative tests for the enzyme were positive with the following materials: pea, pine, and fenugreek seeds, corn germ, potato sprouts, and barley shoots. Negative or non-reproducible results which may be attributed to interfering enzymes were obtained with sugar beet and cane sugar leaves and with beet roots.

DISCUSSION

The equilibrium constant of the sucrose phosphorylase reaction has been found to be 0.053 at pH 6.6 and 30° (4). This displacement in favor of the monosaccharides makes the enzyme appropriate for the utilization of sucrose, and this is probably its main function in *P. saccharophila*. In contrast, the equilibrium of the reaction starting with UDPG and fructose is in favor of sucrose synthesis. Accurate values have not been obtained, but the $\Delta F°$ at 37° can be estimated to be about −1000 as compared to +1770 for sucrose phosphorylase.

The enzyme has been found in many plant materials, and hence we may conclude that it catalyzes a reaction which is fairly general in the plant kingdom. Thus, the enzyme is probably involved in the formation of sucrose by pea extracts detected by Turner (19). However, the synthesis of sucrose from fructose and UDPG is not the only pathway in plants since, as reported in the following paper, another enzyme catalyzes the synthesis of sucrose phosphate from fructose-6-phosphate and UDPG, and the reaction product can be transformed into sucrose by phosphatase action.

According to modern nomenclature (20), the enzyme might be named UDPG-fructose transglycosylase. Following the suggestion of von Euler (21) that enzymes might receive the name of the substrates which they synthesize, with the ending changed to *ese*, another possible name is saccharese. This shorter alternative is used currently in this laboratory.

SUMMARY

A wheat germ enzyme which catalyzes the reaction

$$\text{UDPG} + \text{fructose} \rightleftarrows \text{sucrose} + \text{UDP}$$

has been studied.

Methods for its estimation and purification are described, as well as the conditions for its maximal activity. The equilibrium of the reaction was found to be displaced in favor of sucrose synthesis, the value of K being about 5 at 37° and pH 7.4. The enzyme was detected in several plant tissues.

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THE BIOSYNTHESIS OF SUCROSE PHOSPHATE*

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The enzymic formation of sucrose from UDPG[1] and fructose has been described in the preceding paper (1). While some preparations of the enzyme were found to be almost devoid of action of fructose-6-phosphate, other extracts catalyzed the formation of free sucrose either from fructose or from its phosphate. Since these extracts contain phosphatase, it seemed likely that the action on fructose phosphate was the sum of Reaction 1 plus Reaction 2.

$$\text{Fructose-6-phosphate} + H_2O \rightarrow \text{fructose} + \text{inorganic phosphate} \quad (1)$$

$$\text{UDPG} + \text{fructose} \rightleftarrows \text{sucrose} + \text{UDP} \quad (2)$$

However, further work has shown that it is possible to obtain enzyme preparations which catalyze Reaction 3.

$$\text{UDPG} + \text{fructose-6-phosphate} \rightleftarrows \text{sucrose phosphate} + \text{UDP} \quad (3)$$

Phosphatase acts on sucrose phosphate according to Reaction 4.

$$\text{Sucrose phosphate} \rightarrow \text{sucrose} + \text{phosphate} \quad (4)$$

Crude extracts of wheat germ catalyze all four reactions, and it has not been possible to obtain a complete separation of the enzymes. Nevertheless, with some of the preparations the rate of Reaction 3 was 2 or 3 times faster than that of Reaction 2, and sucrose phosphate has been obtained in amounts which have allowed the determination of its structure with reasonable certainty.

Methods

Preparation of Enzyme—The method of separation of the enzymes acting on fructose and on fructose phosphate was based on the observation that the fructose phosphate enzyme has a greater tendency to become insoluble

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[1] The following abbreviations are used: UDP for uridine diphosphate, UDPG for uridine diphosphate glucose, Tris for tris(hydroxymethyl)aminomethane, and TPN for triphosphopyridine nucleotide.

XUM

when dialyzed against water. The procedure described in the preceding paper was used, but with some changes. The mixing with a blender was omitted, thus making centrifugation at high speed unnecessary, as well as the first dialysis. The wheat germ, 100 gm., was suspended in 300 ml. of 0.05 M phosphate buffer of pH 7.1 and left standing at 5° during 1 hour. The paste was centrifuged 15 minutes at 3000 r.p.m. Without dialyzing, the supernatant fluid was treated with ammonium sulfate, manganous chloride, and ammonium sulfate again, as previously described. The precipitate obtained in the second ammonium sulfate treatment (Fraction IV) was dissolved in the smallest possible amount of water and dialyzed overnight at 5° against several changes of distilled water. The precipitate was

TABLE I

Purification of Enzyme

The activity is expressed in micromoles of total sucrose (free plus esterified) formed in 30 minutes per mg. of protein under the conditions described in the text.

Preparation	Protein	Activity on		$\frac{A}{B}$
		Fructose (*A*)	Fructose-6-phosphate (*B*)	
	mg. per ml.			
Fraction IV	23	1.16	0.20	5.8
1. 0.05 M $(NH_4)_2SO_4$ extract	40	0.70	0.65*	1.06
2. 0.1 " " "	22	0.50	0.90†	0.55
3. 0.1 " " "	10	0.40	1.20	0.33
4. 0.1 " " "	4.4	0.90	1.10	0.82

* and † 65 and 82 per cent, respectively, of the total sucrose formed was esterified.

separated by centrifugation and washed three to four times by suspending in 1 ml. of distilled water and centrifuging. The precipitate was then extracted successively with 2 ml. of 0.05 M and 0.1 M ammonium sulfate. The pooled extracts were dialyzed overnight. The precipitate was washed and extracted successively with 2 ml. of ammonium sulfate at the concentrations shown in Table I (Preparations 1 to 4). In some cases, the precipitation by dialysis and extraction had to be repeated in order to obtain a preparation with a low content in phosphatase and in UDPG fructose transglycosylase.

Analytical—Sucrose phosphate was estimated as described by Cardini *et al.* (1) with a sucrose standard. In order to distinguish sucrose from its phosphate, the samples were analyzed with and without precipitation with the zinc sulfate-barium hydroxide reagent (2). The Fiske and Subbarow (3) method was used for the estimation of phosphate.

Fructose-6-phosphate was estimated by using a preparation of glucose-phosphate dehydrogenase (4), which also contained phosphohexose isomerase.

Substrates—Fructose-6-phosphate was a commercial sample. A synthetic sucrose phosphate was prepared by direct phosphorylation of sucrose as described by Neuberg and Pollak (5).

Fructose-1-phosphate was synthesized according to Raymond and Levene (6).

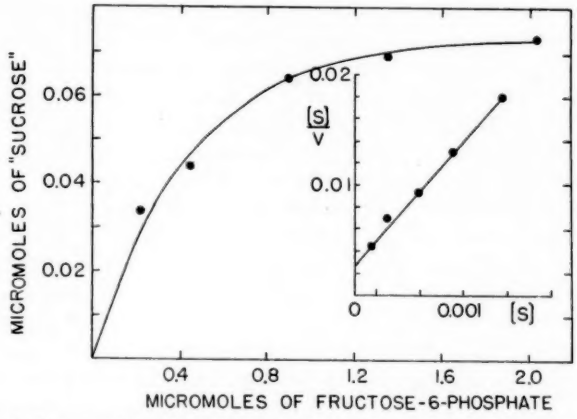

FIG. 1. Influence of fructose-6-phosphate concentration. System as described in the text. The amount of purified enzyme corresponds to 0.05 mg. of protein. Fructose-6-phosphate concentration as indicated. Incubated 30 minutes at 37°. Section at right, a Lineweaver-Burk (18) plot. $K_m = 2.2 \times 10^{-3}$.

Estimation of Enzyme—The procedure was as described in the previous paper, but the pH of the buffer was 6.4 and fructose-6-phosphate was used instead of fructose.

Results

Properties of Enzyme—A comparison of "sucrose" formation from free fructose and fructose-6-phosphate with different preparations of the enzyme has shown that the ratio of activities can vary from 5.8 to 0.33 (Table I), and this is considered proof that two different enzymes are involved. Even the best preparations still contained some phosphatase, and hence the reaction product was usually a mixture of sucrose phosphate and free sucrose. This contamination decreased considerably the yield of sucrose phosphate, thus rendering the isolation more difficult. The substitution of fructose-1-phosphate for fructose-6-phosphate as a substrate for the en-

zyme led to a great decrease in the yield of "sucrose," most of which was free. Nevertheless, there was a small amount which appeared to be esterified, but this point could not be clarified because the sample of fructose-1-phosphate contained a small amount of the 6 ester and because of the presence of phosphatase. This point will have to be investigated further.

The effect of changing the concentration of fructose-6-phosphate is seen in Fig. 1. Calculation of the apparent Michaelis constant gave a value of 2.2×10^{-3}. Fig. 2 shows the influence of changing the pH. The optimum was at pH 6.4.

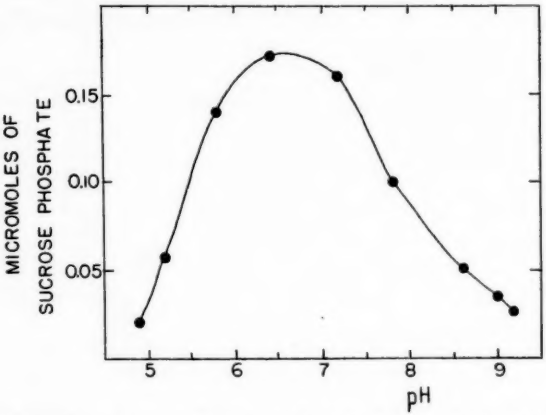

FIG. 2. pH optimum curve. System as described in the text with Tris or acetate buffer at 0.15 M final concentration. Incubated 60 minutes at 37°. The pH was determined on aliquots with a glass electrode.

Isolation of Reaction Product—After many preliminary trials with paper chromatography and electrophoresis, the separation of the reaction product was carried out by anion exchange chromatography. The method of Khym and Cohn (7) with slight modifications was used. The effluents were analyzed by the resorcinol method (8), with and without previous alkaline treatment, and by ultraviolet absorption at 260 mμ. The procedure was as follows: The reaction mixture was prepared by mixing 160 μmoles of UDPG, 500 μmoles of fructose-6-phosphate, and 8 ml. of enzyme (total volume, 40 ml.) and incubating 1 hour at 37°. The proteins were coagulated by heating and filtered. The solution was passed through Dowex 50 and neutralized with ammonia. It contained 40 μmoles of "sucrose phosphate" in 155 ml. This was percolated into a column of Dowex 1 in the chloride form (4.15 sq. cm. × 12 cm.), followed by 300 ml. of 0.001 M ammonia. All the organic phosphate was retained in the

column. Gradient elution was carried out with an apparatus similar to that described by Alm, Williams, and Tiselius (9) by adding 0.03 M ammonium chloride to a 500 ml. mixing chamber filled with a solution containing 0.025 M ammonium chloride and 0.01 M sodium borate. Free sucrose and some ultraviolet-absorbing substances emerged first, and, after 450 ml. had passed through the column, "sucrose phosphate" began to emerge and was collected in 315 ml. Fructose-6-phosphate was retained even after several liters of this solvent had been passed through the column. The tubes containing "sucrose phosphate" were pooled, and the solution was passed through Dowex 50, neutralized with ammonia, and evaporated to dryness under vacuum. The borate was then removed by 3-fold addition of methanol and vacuum distillation. The product was dissolved in water, passed through Dowex 50, and neutralized with calcium carbonate. After filtering, the liquid was evaporated to dryness. Extraction with 95 per cent ethanol removed the calcium chloride, leaving the calcium salt of "sucrose phosphate." The latter was dissolved in 1 ml. of water, clarified by centrifugation, and precipitated with 6 volumes of ethanol, washed with ethanol, and dried. About 17 mg. of a white powder were obtained. The "organic phosphate" content was 1.4 μmoles per mg. (theoretical for the anhydrous Ca salt = 2.44). The fructose-phosphate ratio was 0.90.

Properties of Reaction Product—The product obtained was found to have no reducing power or ultraviolet absorption at 260 mμ. The estimation of fructose by Roe's (8) resorcinol method corresponded to 90 per cent of the organic phosphate. It is known that fructose esters give less color than free fructose; thus Lutwak and Sacks (10) obtained values as low as 58 per cent for fructose-6-phosphate and 87 per cent for fructose diphosphate, and there is always some uncertainty in respect to the purity of the samples.

The substance is stable to alkali. Thus, no phosphate was liberated by heating in 0.5 N alkali during 30 minutes, in contrast to fructose phosphates (Table II). The fructose content estimated by the resorcinol method did not change after heating 10 minutes in 0.2 N alkali during 10 minutes. The rate of removal of the phosphate group by 1 N acid at 100° is similar to that of fructose-6-phosphate and clearly different from that of fructose-1-phosphate and of synthetic sucrose phosphate, which is a mixture of isomers (Table II).

The appearance of reducing power during acid hydrolysis was measured (Fig. 3). Under those conditions, sucrose was hydrolyzed about 5 times faster than the "reaction product." The "reaction product" does not reduce TPN when incubated with glucose dehydrogenase plus isomerase (Table III). However, reduction of TPN takes place with the product of hydrolysis.

The results of experiments by paper chromatography (Table IV) may be summarized as follows: Mild acid hydrolysis gives a glucose spot and a

TABLE II

Acid and Alkaline Hydrolysis

The results represent the per cent of organic phosphate liberated. Alkaline hydrolysis was carried out with sodium hydroxide in silver test-tubes.

	1 N sulfuric acid				0.5 N alkali	
	Heating at 100°					
	10 min.	20 min.	30 min.	60 min.	15 min.	30 min.
Fructose-1-phosphate	77	87	100		92	100
Fructose-6-phosphate			13	22	76	78
Reaction product			10	25	0	0
Synthetic sucrose phosphate			42	52	0	0

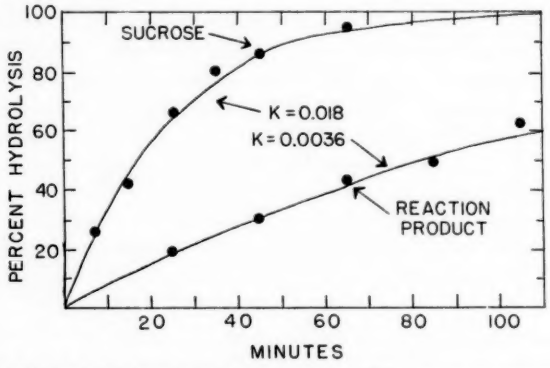

FIG. 3. Acid hydrolysis of reaction product. The curves represent the theoretical values calculated for a first order reaction ($\log_{10}$ and minutes). The points correspond to the results obtained by incubating 0.15 μmole of substance at 37° in 0.9 N hydrochloric acid. After the specified time, the samples were neutralized and the reducing power was measured by the ferricyanide method (19). Samples which were heated 4 minutes at 100° in 0.9 N acid were considered to give the value for 100 per cent hydrolysis.

phosphoric ester spot, but no free fructose. Treatment with kidney phosphatase or with a crude extract of wheat germ in the presence of Mg^{++} results in a substance migrating like sucrose. Hydrolysis of this "sucrose" with acid or with yeast invertase gives glucose and fructose. Other experiments showed that the "reaction product" can be separated from fructose-

6-phosphate by paper chromatography with several solvents. Thus, with methanol-formic acid (11) or with ethanol ammonium acetate of pH 3.8

TABLE III

Reduction of TPN by Reaction Product after Hydrolysis

The substances were mixed with 0.15 μmole of TPN, 0.4 ml. of 1 per cent sodium bicarbonate, 3 mg. of *Zwischenferment*, and water to 2.5 ml. The TPN reduced was calculated from the increase in absorbancy at 340 mμ. Results in micromoles.

Substance	TPN reduced (A)	Substance added* (B)	$\frac{A}{B}$
Fructose-6-phosphate	0.08	0.07	1.14
Fructose-1-phosphate	0.004	0.13	0.03
Reaction product	0.006	0.14	0.04
" " hydrolyzed†	0.068	0.074	0.92

* Calculated from the organic phosphate content.
† Heated 5 minutes at 100° in 0.1 N acid.

TABLE IV

Paper Chromatography of Reaction Product

Solvent, *n*-butanol-pyridine-water, 6:4:1.5. Benzidine-trichloroacetic acid reagent (20) as developer.

	R_{xylose}		
	Experiment I	Experiment II	Experiment III
Reaction product		0.04	
" " + acid hydrolysis*		0.1, 0.75	
" " + kidney phosphatase	0.56		0.59
" " + crude wheat germ	0.55		
" " + kidney phosphatase + invertase			0.76, 0.84
Sucrose	0.56	0.52	0.59
" + invertase			0.76, 0.85
Fructose	0.82	0.83	0.84
Glucose		0.75	0.75
Fructose-6-phosphate		0.04	

* Heated 5 minutes in 0.1 N acid at 100°.

(12), it migrates at about 75 per cent the rate of fructose-6-phosphate. About the same separation could be obtained by paper electrophoresis with borate buffer (13). These procedures were not used for the isolation of the substance because there was overlapping with ultraviolet-absorbing substances.

The reaction product was found to remain unaffected after treatment with yeast or honey invertase. The latter finding was unexpected, since honey invertase (14) is believed to be specific for the glucose moiety of the substrate.

DISCUSSION

It can be concluded that the reaction product is sucrose with a phosphate group at position 6 of the fructose moiety, because dephosphorylation with phosphatase produces a substance behaving like sucrose, and mild acid hydrolysis gives free glucose and a fructose ester which behaves like fructose-6-phosphate when treated with acid or with isomerase plus glucose dehydrogenase. However, it should be pointed out that the samples obtained were only about 60 per cent pure, as judged by the phosphate content of the calcium salt. Further purification was not attempted because only small amounts were available. A point of interest is that sucrose phosphate is more stable to acid than is free sucrose. Thus, the ratio of the hydrolysis constants for the glycosidic links, (K for sucrose)/(K for sucrose phosphate), is 5 in 0.9 N acid at 100°. It was found previously (15) that the ratio of the hydrolysis constants of the phosphate in position 1, (K for glucose-1-phosphate)/(K for glucose-1,6-diphosphate), is 4 in 0.25 N acid at 37°. In both cases, the phosphate group at position 6 stabilizes the substituents at the other end of the molecule.

The sucrose phosphate detected by Buchanan (16) was believed to yield fructose-1-phosphate by hydrolysis, and hence it would be different from the product described in this paper.

A study of the distribution of the two enzymes which lead to the synthesis of sucrose would be of interest for plant physiology, but is difficult to carry out, owing to the presence of interfering enzymes (phosphatase and sucrase). Studies on green leaves with labeled substrates (17) have shown that the labels are introduced into both halves of sucrose before appearing in the free monosaccharides. This would prove that free fructose is not involved directly in the synthesis, were it not for the fact that the same experiments demonstrated that most of the sugars in leaves are stored in a metabolically inert compartment. Thus the introduction of the label in free fructose at the site of synthesis might have been faster than it appeared to be in these experiments.

SUMMARY

A wheat germ enzyme which catalyzes the following reaction has been studied:

UDPG + fructose-6-phosphate → UDP + sucrose phosphate

It is concluded that the reaction product is sucrose with a phosphate group at position 6 of the fructose moiety, because dephosphorylation with phosphatase yields a substance behaving like sucrose and mild acid hydrolysis gives free glucose and a fructose ester which behaves like fructose-6-phosphate when treated with acid or with isomerase, glucose dehydrogenase, and TPN.

The glycosidic linkage of sucrose phosphate was found to be more stable to acid than was that of sucrose.

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THE INFLUENCE OF HORMONAL FACTORS AND OF DIET UPON HEPATIC GLUCOSE-6-PHOSPHATASE ACTIVITY*

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It is well known that the hepatic utilization of glucose is impaired in alloxan diabetes, and evidence exists which suggests that in diabetes the conversion of glucose to glucose-6-phosphate is impaired (1). It has been suggested that the enzyme hexokinase is less active in the diabetic animal (2).

The presence in liver of a specific glucose-6-phosphatase has been demonstrated by Swanson (3) and by de Duve and coworkers (4, 5). Presumably this enzyme is responsible for contributing free glucose to the blood from the liver glycogen pool and from other precursors of glucose-6-phosphate. Since the net rate of formation of glucose-6-phosphate depends upon the relative velocities of the hexokinase and glucose-6-phosphatase reactions, it appeared possible that an increased activity of glucose-6-phosphatase in the liver might be responsible for decreased net utilization of glucose by this tissue, even if the hexokinase reaction were functioning normally. In this paper is presented evidence that there is greatly increased activity of glucose-6-phosphatase in the livers of alloxan-diabetic rats, and that this increased activity appears to be related rather specifically to the diabetic state.

EXPERIMENTAL

Young male rats of the Sprague-Dawley strain weighing 75 to 200 gm. were used as experimental animals in all the experiments. They were maintained on a diet of Rockland rat pellets fed *ad libitum*, except during periods of experimental fasting. Alloxan diabetes was induced by the intravenous injection of 50 mg. of alloxan monohydrate per kilo of body weight. 1 to 7 weeks later animals having blood glucose concentrations (6) in excess of 200 mg. per cent were sacrificed, and their livers were assayed for glucose-6-phosphatase activity.

Quantitative assay for glucose-6-phosphatase activity was carried out as follows. Weighed portions of the livers from both normal and treated animals were homogenized in exactly 3 volumes of 0.25 M sucrose in a

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glass Potter-Elvehjem homogenizer tube with a Teflon pestle. The homogenates were then fractionated into their subcellular components by differential centrifugation (7). The supernatant layer remaining after centrifugation at 600 × g for 10 minutes is referred to as S_1 fraction; the particles not sedimented in 20 minutes at 10,000 × g, but sedimented in 20 minutes at 100,000 × g, are designated as the "microsomal fraction." Low and medium speed centrifugations were carried out in a Servall SS-1A centrifuge, while high speed centrifugations were performed in a Spinco preparative ultracentrifuge, model L. All operations were carried out at 0°. The liver fractions were assayed for glucose-6-phosphatase activity by incubation in 0.08 M citrate buffer, pH 6.5, containing 0.02 M glucose-6-phosphate, for 30 minutes at 30.5°. The assay conditions were so arranged that zero order kinetics were obtained. The reaction was terminated by the addition of an equal volume of 10 per cent trichloroacetic acid, and the inorganic phosphorus which had been liberated by enzymatic hydrolysis was determined by the method of Gomori (8). The values obtained are expressed in two ways in Tables I to IV. The specific activity of each enzyme preparation is expressed as micromoles of organic phosphorus hydrolyzed per minute by the enzyme derived from 1 gm. of liver. In order to compensate for fluctuations in hepatic weight and for the use of animals of varying body weight, in the columns labeled "Total activity" the specific activity of the enzyme has been multiplied by the weight of the whole liver, divided by the body weight of the animal, and multiplied by 100. This calculation normalized values to give the total hepatic glucose-6-phosphatase activity per 100 gm. animal.

The source of materials used was as follows. Barium glucose-6-phosphate heptahydrate was obtained from the Sigma Chemical Company; it was converted to the sodium salt prior to use. Cortisone acetate was a commercial product of Merck and Company. Lipo-adrenal cortex in cottonseed oil was a product of The Upjohn Company. Protamine zinc insulin was a commercial preparation of E. R. Squibb and Sons. Crystallized pituitary growth hormone was a generous gift of Dr. Albert Lehninger.

Results

In Table I are presented data showing the concentration and intracellular distribution of glucose-6-phosphatase activity in normal rat liver preparations. It can be seen that the average rate of hydrolysis of glucose-6-phosphate by the normal liver homogenate is 5.1 μmoles per minute per gm. of wet weight of liver or 23.4 μmoles per minute per 100 gm. of total body weight. Approximately 65 per cent of this activity accompanies the microsomal fraction, which is in accord with the earlier findings of de Duve and Beaufays (4) on the intracellular localization of this enzyme.

The glucose-6-phosphatase activities observed in preparations from the livers of alloxan-diabetic rats are shown in Table II. It is seen that the average activity of these preparations, both per gm. of liver and per 100 gm. animal, is more than double the corresponding values found for normal

TABLE I

Glucose-6-phosphatase Activity in Livers of Normal Rats

Experiment No.	Fraction S_1		Microsomal fraction	
	Specific activity	Total activity	Specific activity	Total activity
1	4.2	18.1	2.9	12.6
2	4.8	23.6	3.4	16.5
3	4.3	22.0	2.9	14.8
4	5.8	25.2	3.5	15.5
5	5.6	24.8	2.8	12.6
6	4.9	20.0	3.5	14.2
7	5.0	23.9	3.4	15.8
8	6.1	29.4	3.9	19.4
9			3.4	12.3
10			3.6	12.3
11			3.6	14.5
12			2.9	11.6
13			3.5	14.5
14			3.4	14.2
15			4.1	15.5
16			2.8	12.2
Average ± s.d.*....	5.1 ± 0.6	23.4 ± 3.2	3.4 ± 0.4	14.3 ± 2.0

The preparation of the S_1 fraction and the "microsomal fraction" is described in the text. Specific activity is in micromoles of phosphorus liberated from glucose-6-phosphate per minute by the enzyme derived from 1 gm. of rat liver. The total activity represents the specific activity multiplied by the total weight of liver divided by the body weight of animal multiplied by 100.

* Standard deviation.

animals. There is no overlapping of values between normal and diabetic animals.

Since a large and reproducible increase in glucose-6-phosphatase activity was found in the diabetic liver, it became of interest to determine whether or not administration of insulin would reverse this effect. In Table III it is seen that the administration of insulin to diabetic rats 12 hours prior to sacrifice resulted in a return of the specific activity values to essentially normal levels and produced a significant lowering of the total activity values.

It also seemed desirable to determine whether an increased glucose-6-

phosphatase activity in the diabetic liver was specifically related to the diabetic state or whether an increased activity could be induced by other

TABLE II

Glucose-6-phosphatase Activity in Livers of Diabetic Rats

Experiment No.	Fraction S_1		Microsomal fraction		Blood glucose
	Specific activity	Total activity	Specific activity	Total activity	
					mg. per cent
1	12.5	46.8	6.7	25.8	325
2	12.0	60.1	9.2	46.5	200
3	11.3	52.6	7.6	35.2	517
4	11.1	47.8	6.5	28.1	475
5	14.2	72.3	9.0	45.5	445
6	11.2	55.2			770
Average ± s.d.....	12.1 ± 1.1	55.8 ± 7.6	7.8 ± 1.1	36.2 ± 8.6	
Normal average ± s.d.*.....	5.1 ± 0.6	23.4 ± 3.2	3.4 ± 0.4	14.3 ± 2.0	

* Average values from Table I.

TABLE III

Effect of Insulin Administration on Glucose-6-phosphatase Activity in Livers of Diabetic Rats*

Experiment No.	Fraction S_1		Blood glucose†
	Specific activity	Total activity	
			mg. per cent
7	4.2	38.4	435
8	5.6	29.9	530
9	5.4	29.9	725
10	6.8	38.1	665
Average ± s.d.....	5.5 ± 0.9	34.1 ± 4.3	
Normal average‡ ± s.d.....	5.1 ± 0.6	23.4 ± 3.2	

* 200 units of protamine zinc insulin per kilo administered subcutaneously 12 hours prior to sacrifice.

† Blood glucose concentrations 3 days prior to insulin injection.

‡ Average values from Table I.

means. Since fasting (9), administration of adrenal cortical hormones (10), and treatment with pituitary growth hormone (11) have each been reported to produce in animals biochemical symptoms resembling those of diabetes,

the effect of these factors on hepatic glucose-6-phosphatase activity was determined. The results of these studies are shown in Table IV. It can be seen that, although fasting of normal rats for 18 to 24 hours results in an

TABLE IV

Effects of Fasting, Adrenal Cortical Hormones, and Pituitary Growth Hormone on Hepatic Glucose-6-phosphatase Activity

	Experiment No.	Fraction S_1		Microsomal fraction	
		Specific activity	Total activity	Specific activity	Total activity
Fasted*	1			5.5	20.3
	2			4.7	13.9
	3			5.1	13.2
	4	8.6	34.5		
	5	5.8	21.9		
	6	8.3	33.5		
Average		7.6	30.0	5.1	15.8
Cortisone†	1			4.5	16.1
	2			4.9	19.3
	3			4.2	16.5
	4			3.7	13.5
	5			4.8	17.4
Average ± s.d.				4.4 ± 0.4	16.6 ± 1.9
Lipo-adrenal cortex‡	1	4.7	23.8	3.8	19.4
	2	5.3	27.1	4.0	21.0
	3	5.3	21.9	3.7	15.5
	4	6.1	26.8	4.6	20.0
	5	5.9	28.1	3.6	17.4
	6	5.5	28.4	3.4	17.4
Average ± s.d.		5.5 ± 0.5	26.0 ± 2.4	3.9 ± 0.4	18.5 ± 1.9
Growth hormone§	1	4.5	22.9		
	2	5.2	27.4		
Normal average ± s.d.		5.1 ± 0.6	23.4 ± 3.2	3.4 ± 0.4	14.3 ± 2.0

* Fasted for 18 to 24 hours preceding sacrifice.

† Cortisone acetate administered intramuscularly daily, 100 mg. per kilo per day for 4 days.

‡ Administered intramuscularly, 5 ml. per kilo, 18 hours prior to sacrifice.

§ Administered intraperitoneally in a single dose, 10 mg. per kilo, 18 hours prior to sacrifice.

average increase of approximately 50 per cent in specific activities, it produces a less significant change in total activities.

The administration of either cortisone or adrenal cortical extract produced modest, unimpressive increases in hepatic glucose-6-phosphatase activities, as shown in Table IV. In two instances, the administration of pituitary growth hormone had no significant effect upon the enzymatic activity.

DISCUSSION

From the results in Table II, it is apparent that alloxan diabetes in the rat is accompanied by a more than 2-fold increase in hepatic glucose-6-phosphatase activity. This is manifested as both an increased tissue concentration of the enzyme and an increased total activity of the enzyme per standard animal. Further, increased activity is observed both in whole liver homogenates and in the microsomal fraction of liver.

That the observed increase in total glucose-6-phosphatase activity in the diabetic liver has some degree of specificity is shown by the fact that several factors which are known to produce metabolic alterations similar to those seen in the diabetic state have relatively little effect upon the total glucose-6-phosphatase activity. For example, cortisone administration has been shown by Welt and coworkers to result in a 7-fold increase in the production of glucose from non-carbohydrate precursors (10). However, neither cortisone in large doses nor crude adrenal cortical extract causes an increase in glucose-6-phosphatase activity comparable to that observed in the diabetic animal. Fasting is known to initiate increased hepatic production of ketone bodies and to impair lipogenesis (9) as does diabetes (12). However, while the fasted animal shows a 50 per cent increase in the concentration of glucose-6-phosphatase in the liver, the increase in total activity per 100 gm. rat is less than one-third that observed in the diabetic animal. Finally, the injection of pituitary growth hormone was without effect upon either the specific activity or total activity of hepatic glucose-6-phosphatase.

While this manuscript was in preparation, Ashmore *et al.* (13) published the results of experiments which also indicated that glucose-6-phosphatase activity is increased in the livers of diabetic animals, and that this enzymatic activity was decreased when insulin was administered over rather prolonged periods. Although a different strain of animals was used in our experiments, the results are essentially in agreement with those of Ashmore and coworkers. In addition, we have determined the effects of other hormonal factors upon hepatic glucose-6-phosphatase activity.

That changes in the concentration of liver enzymes in the diabetic animal may not be limited to glucose-6-phosphatase would seem to be indicated by the results of Copenhaver *et al.* (14). These investigators have

reported that in diabetic liver there is also a 2-fold increase in the specific activity of the glutamic-aspartic transaminase system. Although it is difficult to decide from the data presented by these authors whether the increased specific activity observed was due to an increased total quantity of enzyme in a liver of normal size or whether it was related to a normal quantity of enzyme concentrated in a shrunken liver, the former possibility appears the more likely.

It is not clear whether the increased activity of glucose-6-phosphatase in the diabetic animal is due to an increased total quantity of otherwise fully "activated" enzyme or whether it is the result of an activation or "unmasking" process. However, we have recently obtained results which may bear on this point. Glucose-6-phosphatase in the native state is an enzyme tightly associated with the insoluble microsomal particles. We have succeeded in preparing soluble glucose-6-phosphatase from normal liver microsomes;[1] during the initial step in this procedure a large increase in total activity occurred. It is thus apparent that, in the normal animal, a considerable portion of the activity may be "masked." It is possible that glucose-6-phosphatase is more fully activated in the diabetic liver; this possibility is now being explored.

It is of course not revealed by these experiments whether the activity of hepatic glucose-6-phosphatase is the direct site of action of insulin. However, the fact that insulin administration lowered the activity of the enzyme to nearly normal values within 12 hours would seem to indicate that this is a possibility which must be seriously entertained. Although preliminary attempts to demonstrate an inhibitory action of insulin on glucose-6-phosphatase *in vitro* have been unsuccessful, further work along this line is now in progress.

The possible physiological significance of an increased activity of glucose-6-phosphatase in the liver is readily apparent. Since this enzyme in effect drains off the product of the hexokinase reaction, an increased activity could lead to decreased glucose utilization, even if hexokinase activity in liver is unaltered. Assuming that the enzyme acts at the same rate *in vivo* as it does *in vitro*, it can be calculated that in the liver of the normal 100 gm. rat approximately 6 gm. (33 mmoles) of glucose could be formed from glucose-6-phosphate in 24 hours. In the liver of a comparable diabetic animal, approximately 15 gm. (81 mmoles) of glucose could be formed from glucose-6-phosphate daily. This order of activity would seem to be of sufficient magnitude to suggest that the enzyme may well be at least partially responsible for a decreased hepatic utilization of glucose in the diabetic animal.

[1] D. R. Weakley and R. G. Langdon, to be published.

SUMMARY

1. Dietary and hormonal factors have been examined for their effect on hepatic glucose-6-phosphatase activity.

2. Glucose-6-phosphatase activity has been found to be greatly increased in the liver of the alloxan-diabetic rat. Insulin *in vivo* decreased this elevated activity.

3. The physiological significance and possible mechanism for these effects have been discussed.

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AN ANALYSIS OF THE PUTRESCINE REQUIREMENT OF HEMOPHILUS PARAINFLUENZAE*

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Putrescine is an essential growth factor for *Hemophilus parainfluenzae* 7901 (1, 2) and for *Neisseria perflava* (3). A tetramethylenediamine nucleus was believed to be the essential metabolic requirement of *H. parainfluenzae*, since agmatine, spermine, and spermidine could replace putrescine (2), but more recently 1,3-propanediamine was identified as an equally effective replacement for putrescine (4). Therefore, either a trimethylenediamine or tetramethylenediamine nucleus can be utilized, but both lower and higher homologues of these diamines, *e.g.*, ethylenediamine and cadaverine, are inactive as growth factors (2). This report extends our knowledge of the specificity of this bacterial growth factor requirement and includes a survey of inhibitors of putrescine metabolism.

EXPERIMENTAL

Stock Culture—H. parainfluenzae, ATCC 7901, was carried by weekly transfers on North gelatin agar (Difco) supplemented with 0.1 per cent Bacto-hemoglobin and 0.0001 per cent diphosphopyridine nucleotide (DPN). The cultures were incubated and stored at 34°.

Medium—The composition of the basal medium is shown in Table I. The complex mixture does not support any growth of the organism unless supplemented with putrescine (or its equivalent).

Inoculum—A loop of growth was transferred from a 24 hour culture on North gelatin agar to an agar slant consisting of the medium of Table I supplemented with 0.01 μmole per ml. of putrescine dihydrochloride and solidified by the addition of 1.5 per cent Bacto-agar. After 24 hours of incubation at 34°, a loop of growth was transferred from this agar culture to each of two test-tubes containing 4 ml. of basal medium supplemented with 0.02 μmole per ml. of putrescine dihydrochloride. The tubes were

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incubated in an inclined position for 16 hours at 34°, after which the cells were sedimented by centrifugation and resuspended with sufficient sterile M/30 phosphate buffer, pH 7.3, to permit 80 per cent transmission of incident light (Evelyn colorimeter, 660 mμ filter; 18 mm. tube; M/30 phosphate buffer = 100). This suspension was diluted with an equal volume of sterile M/30 phosphate buffer containing DPN (previously sterilized by filtration through a bacterial filter). 0.1 ml. of the diluted suspension was added to each tube of autoclaved medium.

TABLE I

*Composition of Basal Medium**

Component	mg. per ml.	Component	γ per ml.
*Amino acids**		*Vitamins*	
DL-Alanine	1	Thiamine chloride	1
DL-Aspartic acid	1	Riboflavin	0.1
L-Glutamic acid	1	Pyridoxine·HCl	2
DL-Methionine	0.2	Ca pantothenate	1
DL-Isoleucine	0.2	Niacinamide	0.5
L-Leucine	0.1	Niacin	0.5
DL-Threonine	0.2	Inositol	20
DL-Serine	0.2	Choline chloride	5
L-Proline	0.1	Biotin	0.001
DL-Tryptophan	0.2	*p*-Aminobenzoic acid	0.001
DL-Valine	0.2	Folic acid	0.01
DL-Phenylalanine	0.2	*DPN*	0.25
L-Histidine·HCl	0.1	*Purines-uracil*	
L-Cystine	0.1	Guanine·HCl	10
L-Tyrosine	0.1	Adenine sulfate	10
L-Arginine·HCl	0.2	Uracil	10
L-Lysine·HCl	0.2	*Salts*	
Glycine	0.1	Ca^{++} ($CaCl_2 \cdot 2H_2O$)	1
Glucose	2	Mg^{++} ($MgSO_4 \cdot 7H_2O$)	10
Sodium nitrate	1	Fe^{++} ($FeSO_4 \cdot 7H_2O$)	2.5
" *acetate·3H₂O*	6		
K_2HPO_4	3.5		

* The components in italics are the separate stock solutions. The refrigerated stocks were combined in double strength proportions and adjusted to pH 7.8 with 25 per cent NaOH prior to each experiment; 2 ml. quantities were distributed in 18 × 150 mm. Pyrex test-tubes; test materials and distilled water were added to a final volume of 4 ml. After autoclaving for 15 minutes at 120°, the tubes were stored overnight in the cold, inoculated the following morning (sterile DPN was added with the inoculum), and incubated in an inclined position in test-tube baskets for 48 hours at 34°. Growth was then estimated turbidimetrically with the Evelyn colorimeter.

Putrescine Analogues—All of the *N*-alkyl analogues of putrescine and the corresponding derivatives of 2-butene-1,4-diamine were synthesized in the chemistry department of the University of Connecticut by procedures that either have been published (5) or will be described elsewhere. The sources of other chemicals are indicated in Tables II and III.

Aqueous solutions of all the compounds were passed through sintered glass bacteriological filters and added aseptically to the sterile basal medium.

Results

Growth Factors—The compounds which promote the growth of *H. parainfluenzae* when added to the basal medium are arranged in the order of decreasing activity in Table II. The twenty compounds, all derivatives of 1,3-propanediamine and 1,4-butanediamine (putrescine), show only one other common requirement for growth factor activity; at least one of the two amino groups of the diamine nucleus must be unsubstituted.

The inactivity of ethylenediamine, cadaverine, ornithine, pyrrolidine, 4-amino-1-butanol, and 1,4-butanediol as growth factors (2) has been verified at concentrations up to 0.5 μmole per ml. In addition, histamine, pyrrole, and L- and D-2,4-diaminobutyric acid[1] were inactive at this relatively high concentration. None of these compounds was toxic when subjected to the inhibitor analysis described below.

Inhibitors—We have arbitrarily classified as inhibitors those structural analogues of putrescine and 1,3-propanediamine which inhibit growth of *H. parainfluenzae* 100 per cent in the presence of 0.028 μmole per ml. of putrescine. Furthermore, only those compounds which were inhibitory when present at 10 μmoles per ml. or less were so classified. In Table III the relative toxicity of one group of these derivatives is illustrated. Curiously, the most active growth factor (Table II), *N*-(3-aminopropyl)-1,3-propanediamine, also heads this list of inhibitors. The antibiotic, nocardamine, isolated and characterized by Stoll and his coworkers (6), was included in this study although it is a derivative of the inactive compound, cadaverine. Arcaine, an inhibitor of mammalian diamine oxidase (7) and bacterial diamine oxidase (8), was also an inhibitor in our system. However, isoniazid and iproniazid, the antituberculosis drugs which are strong inhibitors of diamine oxidase (9) and the plasma amine oxidase (10), did not inhibit growth of *H. parainfluenzae*.[2]

In Table IV we have summarized the results of a systematic study of *N*-alkyl analogues of putrescine as inhibitors of the growth of *H. parain-*

[1] We wish to thank Dr. Jesse P. Greenstein, National Institutes of Health, Bethesda, for these isomers.

[2] E. J. Herbst and E. B. Glinos, to be published.

fluenzae.[3] In two instances, namely with *N*-*n*-butyl-1,4-butanediamine and *N*-*n*-propyl-1,4-butanediamine, the phenomenon of relatively high growth factor activity and concomitant high toxicity (previously cited in

TABLE II

Comparative Activity of Growth Factors for H. parainfluenzae

Compound No.*		Concentration†
		μmole per ml.
1	$NH_2(CH_2)_3NH(CH_2)_3NH_2$	0.001
2	$NH_2(CH_2)_3NH(CH_2)_4NH_2$	0.0015
3	$NH_2(CH_2)_4NH_2$	0.0015
4	$NH_2(CH_2)_4NH(CH_2)_4NH_2$	0.0016
5	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	0.002
6	$NH_2(CH_2)_3NH_2$	0.0024
7	$NH_2CH_2CHOHCH_2NH_2$	0.0025
8	$NH_2C(:NH)NH_2(CH_2)_4NH_2$	0.0037
9	$CH_3NH(CH_2)_4NH_2$	0.004
10	$NH_2CH_2CH{=}CHCH_2NH_2$	0.008
11	$NH_2(CH_2)_3NH(CH_2)_3NH_2$	0.009
12	$CH_3CH_2NHCH_2CH{=}CHCH_2NH_2$	0.011
13	$CH_3(CH_2)_2NH(CH_2)_4NH_2$	0.014
14	$(CH_3)_2N(CH_2)_4NH_2$	0.03
15	$CH_3NHCH_2CH{=}CHCH_2NH_2$	0.031
16	$(CH_3)_2N(CH_2)_3NH_2$	0.035
17	$CH_3CH_2NH(CH_2)_4NH_2$	0.05
18	$NH_2CH_2CH_2CH(NH_2)CH_3$	0.06
19	$(C_2H_5)_2NCH_2CHOHCH_2NH_2$	0.082
20	$CH_3(CH_2)_3NH(CH_2)_4NH_2$	0.083

* Compounds 1, 2, and 11 were synthesized by Dr. Aldo M. Pulito, The Ames Laboratories, Inc., South Norwalk, Connecticut. Compounds 3 and 19 were Eastman products, while Compounds 5 and 8 were obtained from Hoffmann-La Roche and Company. We wish to thank the following for generous samples: Dr. E. A. Zeller, Northwestern University, Chicago, Compounds 4 and 7; Dr. Charles F. Huebner, Ciba Research Department, Summit, New Jersey, Compound 16; and Dr. P. G. Smith, Sharples Chemicals, Inc., Compounds 6 and 18. Compounds 9, 10, 12, 13, 14, 15, 17, and 20 were prepared in the Chemistry Department at the University of Connecticut.

† Produces half maximal growth.

the case of *N*-(3-aminopropyl)-1,3-propanediamine) is again observed. The growth factor activity of the *N*-ethyl and *N*-methyl analogues is high, but these compounds are not inhibitors. Similarly, the *N*-ethyl and *N*-methyl derivatives of 2-butene-1,4-diamine are non-inhibitory but can function as *H. parainfluenzae* growth factors. In general, the methyl and ethyl

[3] A report of this work was presented at the April, 1952, meeting of the American Society of Biological Chemists (4).

analogues are tolerated at higher concentrations than are the *n*-propyl and *n*-butyl derivatives and the *N*,*N*,*N*′,*N*′-tetra-*n*-butyl compounds are most inhibitory.

TABLE III

Compounds Inhibiting Growth of H. parainfluenzae

Compound No.*		Concentration†
		μmoles per ml.
1	*N*-(3-Aminopropyl)-1,3-propanediamine‡	0.6
2	Nocardamine	1.3
3	Arcaine	1.4
4	*N*,*N*,*N*′,*N*′-Tetraisopropyl-1,3-propanediamine	3
5	*N*,*N*-Di-*n*-propyl-1,3-propanediamine	4
6	N^1,N^1-Diethyl-1,4-pentanediamine	4
7	*N*,*N*-Diisopropyl-1,3-butanediamine	4.4
8	*N*,*N*-Diethyl-1,3-propanediamine	5.8
9	*N*,*N*-Dimethyl-1,3-propanediamine‡	6.2
10	Agmatine‡	9.6

* We wish to thank Dr. A. Stoll, Pharmazeutisch-chemisches Laboratorium, Sandoz, Basel, for the sample of nocardamine. We thank Dr. Charles F. Huebner, Ciba Research Department, for Compounds 5, 8, and 9 and Dr. Thomas M. Laakso, Eastman Kodak Company, Rochester, New York, for Compounds 4 and 7.

† Inhibits growth 100 per cent when tested in the presence of 0.028 μmole per ml. of putrescine.

‡ The compound has growth factor activity at low concentrations (Table II).

TABLE IV

Comparison of Analogues of Putrescine As Inhibitors

Type of analogue	Concentration of analogue for 100 per cent inhibition of growth*			
	R = *n*-butyl	R = *n*-propyl	R = ethyl	R = methyl
	μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*	*μmoles per ml.*
$RNH(CH_2)_4NH_2$	1.7†	4.8†	†	†
$RNHCH_2CH{=}CHCH_2NH_2$	1.7	4.9	†	†
$R_2N(CH_2)_4NH_2$	2.5	4.4	5.2	†
$R_2NCH_2CH{=}CHCH_2NH_2$	1.6	3.7	2.6	8.8
$RNH(CH_2)_4NHR$				6.5
$RNHCH_2CH{=}CHCH_2NHR$	3.2			3.3
$R_2N(CH_2)_4NR_2$	0.8	4.9	5.0	6.9
$R_2NCH_2CH{=}CHCH_2NR_2$	0.4	1.5	3.2	

* In the presence of 0.028 μmole per ml. of putrescine.

† The compound has growth factor activity at low concentrations (Table II).

Reversal of Inhibitors—Complete reversal of the inhibitors can be accomplished by two procedures: (1) increasing the concentration of putrescine

in the medium or (2) replacing putrescine with an equimolar or lower concentration of 1,3-propanediamine, spermidine, or spermine. Reversal of the inhibitory *n*-butyl series of putrescine analogues by putrescine and by 1,3-propanediamine, spermidine, and spermine is illustrated in Table V.

TABLE V

Reversal of n-Butyl Series of Inhibitors

Inhibitor (R = *n*-butyl)	Concentration of inhibitor	Concentration of reversing agent*			
		Putrescine	1,3-Propanediamine	Spermidine	Spermine
	μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*	*μmole per ml.*	*μmole per ml.*
$RNHCH_2CH{=}CHCH_2NH_2$	1.7	0.28	0.032	0.017	0.012
$RNH(CH_2)_4NH_2$	1.7	2.8	0.032	0.017	0.12
$RNHCH_2CH{=}CHCH_2NHR$	3.2	2.8	0.32	0.17	0.12
$R_2NCH_2CH{=}CHCH_2NH_2$	1.6	2.8			
$R_2N(CH_2)_4NH_2$	2.5	2.3	1.0	0.017	0.12
$R_2NCH_2CH{=}CHCH_2NR_2$	0.5	6.2	6.2	†	†
$R_2N(CH_2)_4NR_2$	0.8	5.0	2.5	†	†

* Produces maximal growth in the presence of the inhibitor.
† The compound does not antagonize the inhibitor.

TABLE VI

Reversal of Increments of N,N-Di-n-butylputrescine

Concentration of inhibitor	Concentration of reversing agent*		
	Putrescine	1,3-Propanediamine	Spermidine
μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*	*μmole per ml.*
1.8	0.57	0.07	0.009
2.5	2.3	1.0	0.017
3.6	5.7	10.0	†
5.0	14.0	17.0	†

* Produces maximal growth in the presence of the inhibitor.
† The compound does not antagonize the inhibitor.

Relatively high concentrations of putrescine, sometimes in excess of the inhibitor concentration, were required to neutralize the effect of the mono- and di-*n*-butyl analogues. 1,3-Propanediamine was a more effective reversing agent, while spermine, and especially spermidine, antagonized these inhibitors at low concentrations not much in excess of the growth factor requirement in the absence of inhibitors. The highly toxic tetra-*n*-butyl inhibitors were reversed only by extremely high concentrations of the diamines, while the polyamines were completely ineffective as antagonists.

The action of the inhibitors is not typical competitive inhibition. The application of "inhibition analysis" (11) to these systems has consistently failed to yield constant molar ratios of inhibitor to reversing agent; the pattern of the reversal of increments of *N*,*N*-di-*n*-butylputrescine illustrated in Table VI is representative of results with other inhibitors. The inhibitor-polyamine relationship is very complex. Often a partial reversal of an inhibitor occurs in the presence of moderately low concentrations of polyamine, the degree of reversal not being improved with further small additions of polyamine. When a concentration of 0.5 to 1 μmole per ml. of spermine or spermidine is reached, a reversion to 100 per cent inhibition occurs. This apparent toxicity of relatively low levels of spermine or spermidine is only observed in the presence of inhibitors; when tested alone, the polyamines are non-toxic; *i.e.*, they permit maximal growth of *H. parainfluenzae* at concentrations up to 10 μmoles per ml.

DISCUSSION

Several facts about the nature of the unusual bacterial growth factor requirement for the trimethylenediamine or tetramethylenediamine nucleus are evident from this study. High specificity is indicated by the inactivity of ethylenediamine and cadaverine. However, alteration and substitution in the basic carbon chain are tolerated to a certain extent (Table II). For example, 1,3-diamino-2-propanol is just as active as 1,3-propanediamine, and 2-butene-1,4-diamine is one-sixth as active as putrescine. With α-carbon substitution as in 1,3-butanediamine the activity drops more sharply.

Although *N*-alkyl derivatives of putrescine have growth factor activity (Compounds 9, 13, 17, and 20, Table II), their utilization, relative to that of putrescine, is quite inefficient. Even *N*,*N*-dialkyl substitution of the basic molecule is permissible (Compounds 14 and 16, Table II), but the reduced activity of all these compounds is evidence of the biological preference for the parent molecule. The high activity of *N*-aminoalkyl derivatives of putrescine and 1,3-propanediamine, *e.g.*, spermidine and *N*-(3-aminopropyl)-1,3-propanediamine, is subject to two explanations: either these derivatives are utilized *per se* as efficiently as the diamines, or they are quantitatively converted by amine oxidase (10) to the corresponding diamine. The amine oxidase route seems most likely, but every attempt to show amine oxidase activity in *H. parainfluenzae* preparations has given completely negative results.[4] The high activity of *N*,*N*′-(3-aminopropyl)-1,4-butanediamine (spermine) is more surprising, since *N*,*N*′-dimethyl-1,4-butanediamine is completely inactive as a growth factor as is the corresponding 2-butene derivative. Spermine activity must, therefore,

[4] D. Hubbard, R. Weaver, and E. J. Herbst, to be published.

depend upon the direct and nearly equivalent utilization of the polyamine in the putrescine-dependent metabolism. Another possible explanation of diamine and polyamine equivalence depends upon the biosynthesis of polyamines from the precursor diamines.

$$NH_2(CH_2)_3NH_2 + NH_2(CH_2)_3X \rightarrow NH_2(CH_2)_3NH(CH_2)_3NH_2 + HX$$

$$NH_2(CH_2)_4NH_2 + NH_2(CH_2)_3X \rightarrow NH_2(CH_2)_4NH(CH_2)_3NH_2 + HX$$

$$NH_2(CH_2)_4NH_2 + 2NH_2(CH_2)_3X \rightarrow NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2 + 2HX$$

However, absolutely no evidence supporting this concept of the polyamines as the functional molecules is available. Indirect evidence refuting the polyamines and in support of putrescine as the key compound has been obtained. Five batches of cells of *H. parainfluenzae* grown in the basal media containing either 1,3-propanediamine, putrescine, spermine, spermidine, or agmatine were hydrolyzed; the hydrolysates were examined by paper chromatography with the solvent system ethyleneglycol monomethyl ether-propionic acid-water, 70:15:15. Only one active compound, identified by the bioautographic technique (12) and having an R_F corresponding to that of putrescine in this solvent, was present in each of the five cell hydrolysates. The conversion by *H. parainfluenzae* of spermidine and spermine to putrescine apparently proceeds by a mechanism not involving amine oxidase. This reaction is completely undefined, and the more complex conversion of 1,3-propanediamine to putrescine is equally baffling.

We believe that the mechanism of action of the *N*-alkylputrescines and of the other inhibitory molecules disclosed by this investigation is not a simple interference with putrescine utilization. Several lines of evidence support the concept of multiple interference with *H. parainfluenzae* metabolism involving (*a*) a specific interference at the putrescine site of action and (*b*) a non-specific interference at other cell sites. The pattern of reversal of the inhibitors by putrescine, 1,3-propanediamine, spermine, and spermidine is consistent with this reasoning. At low toxic concentrations of the inhibitors complete reversal is accomplished by moderately low concentrations of putrescine, while in the presence of 1,3-propanediamine, spermidine, or spermine the action of the inhibitors appears to be noncompetitive. These results suggest a specific interference with putrescine utilization only when the molecule is supplied preformed. This picture is altered as the concentration of inhibitor is increased. Extremely high concentrations of putrescine and 1,3-propanediamine, in excess of the inhibitor concentration, are now required to reverse the inhibition while spermidine and spermine are completely ineffective as antagonists. In this area of inhibitor action the low ratios of inhibitor to diamine antagonist are not indicative of a specific action upon diamine-dependent reactions.

Rather, a "non-specific competitive inhibition" (13) involving an ion

exchange phenomenon wherein the basic inhibitors are displaced from vital anions of the bacterium by the diamine cations appears to be operative. The reversal of Atabrine, quinine, and propamidine by spermine, spermidine, and other polyamines (14, 15) has been classified as non-specific competitive inhibition (13), and Albert (16) has linked the antibacterial action of acridines to the blocking of normal metabolic functions of cell anions by the acridine cations. There is evidence that the anion involved in the acridine bacteriostasis is cellular nucleic acid (17). The inhibitors we have studied are all basic molecules and a mechanism of bacteriostasis by non-specific competitive inhibition involving a cell anion is likely. The pattern of reversal of high levels of the inhibitors by equimolar and higher concentrations of the diamines supports this hypothesis. Competition for the cell anions between *N*-alkylputrescines and putrescine would favor the inhibitors, since the basicity of the nitrogen atoms has been increased by the alkyl substitution. The inactivity of spermidine and spermine as antagonists of this non-specific inhibition is unusual, since these polyamines are efficient antagonists of basic drugs (14, 15). However, the apparent toxicity of spermine and spermidine in the presence of the *N*-alkylputrescines complicates the study of the polyamine-inhibitor relationship.

SUMMARY

Culture methods for the demonstration of the putrescine requirement of *Hemophilus parainfluenzae* are described in detail. Twenty compounds, all of which are derivatives of 1,3-propanediamine or 1,4-butanediamine (putrescine), have growth factor activity. Among the active compounds are a number of *N*-alkyl and *N*,*N*-dialkyl derivatives of putrescine; however, the *N*,*N*′-dialkyl and *N*,*N*,*N*′, *N*′-tetraalkyl analogues of putrescine are growth inhibitors. The *n*-butyl derivatives are more toxic than the *n*-propyl-, ethyl-, or methyl-substituted analogues.

Low toxic levels of the inhibitors are efficiently reversed by putrescine and other growth factors, but constant inhibitor-growth factor ratios are not observed. High toxic levels of the analogues are reversed only by massive concentrations of putrescine and 1,3-propanediamine and evidence in support of non-specific inhibitor action is discussed.

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FREE AMINO GROUPS AND *N*-TERMINAL SEQUENCE OF RABBIT ANTIBODIES*

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The character of antibodies as serum globulins is regarded as definitely established, but the chemical relationship of antibody to normal γ-globulin is still in question (1, 2). All of the various theories which have been proposed to account for the synthesis of antibody fall into two basic groups with respect to the nature of the antibody molecule. According to one view, it may be expected that antibody is identical with a normal, nonimmune γ-globulin with respect to amino acid composition and sequence of amino acid residues in the polypeptide chains, and that normal and antibody globulins differ only in the possession by the latter of a surface configuration which can react specifically with antigen. According to the other view, the presence of antigen modifies the apparatus of globulin synthesis so that a different type of molecule is elaborated; it would be expected to differ in amino acid composition and in sequence of residues of the polypeptide chains.

Only fragmentary and indecisive early information is available concerning the amino acid composition of rabbit antibodies (1). However, Porter (3) has demonstrated that normal rabbit γ-globulin and antiovalbumin are alike in possessing the *N*-terminal tetrapeptide sequence, alanylleucylvalylaspartyl, with glutamic acid probably in the 5 position. His results thus indicate that the 2 molecules do not differ in at least a portion of their structure. Such a finding is obviously of great importance and merits extension. Determination of the complete structure of a rabbit antibody molecule is not yet feasible. However, it is desirable to ascertain whether other types of rabbit antibodies possess the same *N*-terminal sequence. The present paper reports studies of the free amino groups of eight different types of antipneumococcal antibodies and the *N*-terminal sequence of four of them. No differences in free amino groups or *N*-terminal sequence could be found. The pentapeptide sequence, alanylleucylvalylaspartylglutamyl, has been definitely established in the antipneumococcal anti-

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bodies and is identical with the sequence found by Porter for normal rabbit γ-globulin and antiovalbumin.

The accompanying paper (4) deals with the amino acid composition of four rabbit antibodies and discusses the over-all significance of the present studies.

Materials and Methods

Antibodies—The rabbit antibody preparations which were used in this and in the accompanying study (4) were concentrated and purified preparations which were prepared between 1938 and 1940 in the laboratories of E. R. Squibb and Sons, and which had been kept in the frozen state since that time. We are greatly indebted to Mr. Tillman D. Gerlough of E. R. Squibb and Sons for his generosity in donating these preparations, in permitting us to describe the method of purification of these antibodies, and in supplying us with the type-specific polysaccharides.

The antibody preparations were obtained by the following procedure. Hyperimmune rabbit plasma was diluted with an equal volume of distilled water, brought to 0.27 saturation with solid ammonium sulfate, and heated at 56° for 30 minutes. The insoluble fraction was discarded and the antibodies were precipitated at 0.5 saturated ammonium sulfate; the precipitate was redissolved in water and reprecipitated at 0.45 saturation. The precipitate was then dialyzed in the cold against water maintained at pH 5.3 and the insoluble fraction was discarded. The supernatant fluid was adjusted to pH 7, and the antibody fraction was collected between 0.43 and 0.47 saturation with ammonium sulfate. The precipitate was then dialyzed against isotonic saline and preserved with 0.25 per cent phenol and a 1:10,000 solution of merthiolate.

When the antibody solutions were thawed, it was found that they contained about 20 per cent protein. Each preparation was examined in the Tiselius apparatus and in the Spinco ultracentrifuge by the usual methods. The medium was a Veronal buffer of 0.1 ionic strength at pH 8.5 to 8.6. The results, given in Table I, show that these globulins are, in general, of high purity and have the expected properties for rabbit γ-globulins. The main sedimenting component corresponds to a particle of about 160,000 molecular weight, as was found earlier for this species (5). A small amount of heavier material, which presumably represents a dimer, has frequently been detected in γ-globulin preparations obtained by chemical fractionation (6, 7).

The amount of specifically precipitable antibody and the quantity of polysaccharide antigen bound in the antigen-antibody complex at the zone of equivalence were measured by the quantitative precipitation methods of Heidelberger and Kendall (8). The results of these studies, given in

Table II, show that the precipitable antibody represents from 14 to 30 per cent of the total protein. In each instance, the precipitation of antibody by antigen followed all the expectations for a system containing a single type of antibody and antigen. The type-specific polysaccharides, referred to as SI, SII, etc., were highly purified preparations obtained from E. R. Squibb and Sons.

TABLE I

*Properties of Purified Rabbit Antibody Preparations**

Electrophoresis and sedimentation were performed on the same solutions containing Veronal (diethyl barbiturate) buffer of 0.1 ionic strength at pH 8.5 to 8.6. The amount of electrophoretic γ-globulin is given as the percentage of the total migrating area, as observed at 1.5° in an apparatus with the schlieren scanning device. Mobility ($-u \times 10^5$) is given in $cm.^2\ volt^{-1}\ sec.^{-1}$. Sedimentation was studied in a Spinco ultracentrifuge at room temperature. The sedimentation constants ($s_{20,w}$) are given in Svedberg units (S) corrected to water and 20°.

γ-Globulin	Concentration	Electrophoretic behavior		Sedimentation behavior			
		$u \times 10^5$	Amount of γ component	Component 1		Component 2	
				$s_{20,w}$	Amount	$s_{20,w}$	Amount
	per cent		*per cent*	*S*	*per cent*	*S*	*per cent*
I	1.25	1.7	99	6.3	92	8.3	8
II	1.25	2.0	99	6.3	93	9.0	7
III	1.03	1.8	99	6.3	92	9.1	8
IV	1.20	1.8	87†	6.2	92	9.1	8
V	0.87	1.7	89	6.5	91	9.2	9
VII	0.87	1.8	87	6.3	92	8.8	8
VIII	0.82	1.9	85	6.3	91	8.8	9
XIV	1.03	1.9	89	6.3	93	10.1	7

* We are indebted to Mr. Douglas M. Brown for his help with these determinations.

† For the last five preparations, the protein other than γ-globulin comprised 3 to 4 per cent albumin and material which spread in mobility between the albumin and the γ-globulin.

The studies of the free amino groups and the *N*-terminal peptide sequence reported herein, and of the amino acid composition of the antibodies in the accompanying paper (4), were performed on the antigen-antibody precipitates obtained at the zone of equivalence. A quantity of 0.005 per cent solution of specific polysaccharide, calculated from the data in Table II, was added to a diluted preparation of the antibody solution and was allowed to stand at 4° until precipitation was complete. The preparation was then centrifuged and the supernatant fluid discarded. The antigen-antibody complex was washed by resuspension in saline and

collection by centrifugation. It was washed finally several times each with deionized water, absolute ethanol, and ether, and was then allowed to dry in the air at room temperature for several days.

Precipitated antibody is designated as antibody I, II, etc. In some cases, the residual protein was precipitated with ethanol and washed and dried as described; such a preparation is γ-globulin I to indicate the origin of the γ-globulin from rabbits immunized against type I pneumococcus. Similarly, γ-globulin M is a mixture of γ-globulins VIII and XIV.

TABLE II

*Immunological Properties of Antibody Preparations**

The equation of Heidelberger and Kendall (8) was used to characterize the antibodies. The equation is (Ab/An) = [2R − (R^2An)/A] where Ab = antibody precipitated, An = antigen added, A = antibody precipitated at the equivalence point, and R = Ab/An at the equivalence point. This equation was followed in the region of antibody excess. Straight lines were obtained in plots of Ab/An *versus* An. At the equivalence point, neither antigen nor antibody was present in the supernatant fluid. In the region of excess antigen, a decrease in precipitate was observed.

Type No.	R	R^2/A	Composition of ppt. at point of equivalence		An per 100 gm. of ppt.	Amount of A per 100 gm. of γ-globulin
			A	An		
			mg.	*mg.*	*gm.*	
I	15.1	153	1.49	0.098	6.2	14.9
II	28.0	240	3.24	0.116	3.5	28.8
III	25.0	300	2.08	0.083	3.8	20.2
IV	16.5	150	1.83	0.110	5.6	19.4
V	20.0	275	1.45	0.073	3.3	15.4
VII	18.5	208	1.65	0.089	5.0	14.4
VIII	23.0	159	3.00	0.130	4.1	30.0
XIV	21.6	191	2.44	0.113	4.4	23.5

* We are indebted to Dr. B. V. Jager for his advice and help with these determinations.

Whole γ-globulin, designated γ-globulin N, was isolated in this laboratory from freshly clotted, normal rabbit blood by the procedure of Kekwick (9); this preparation was shown by electrophoresis at pH 8.6 in Veronal buffer to contain more than 95 per cent γ-globulin.

Preparation and Chromatography of DNP Derivatives—The proteins or protein-polysaccharide complexes were treated with 1,2,4-fluorodinitrobenzene (DNFB) by the method of Sanger (10, 11). The yellow dinitrophenylated proteins (DNP proteins) were washed three times each with water, absolute ethanol, and ether. The amount of protein in the air-dried DNP proteins was estimated by measuring its amide content by the procedure given by Thompson (12). The amide content of anhydrous

γ-globulin M was found to be 1.30 gm. of NH_3 per 100 gm. of protein. In view of the apparent identity of composition of rabbit antibodies (4), it was assumed that the amide content of all of the antibody and γ-globulin preparations is the same. The amount of anhydrous protein in the various preparations of DNP antibodies and γ-globulins was found to be 73 to 78 per cent of the weight; this variation is undoubtedly due to differences in content of polysaccharide and in moisture.

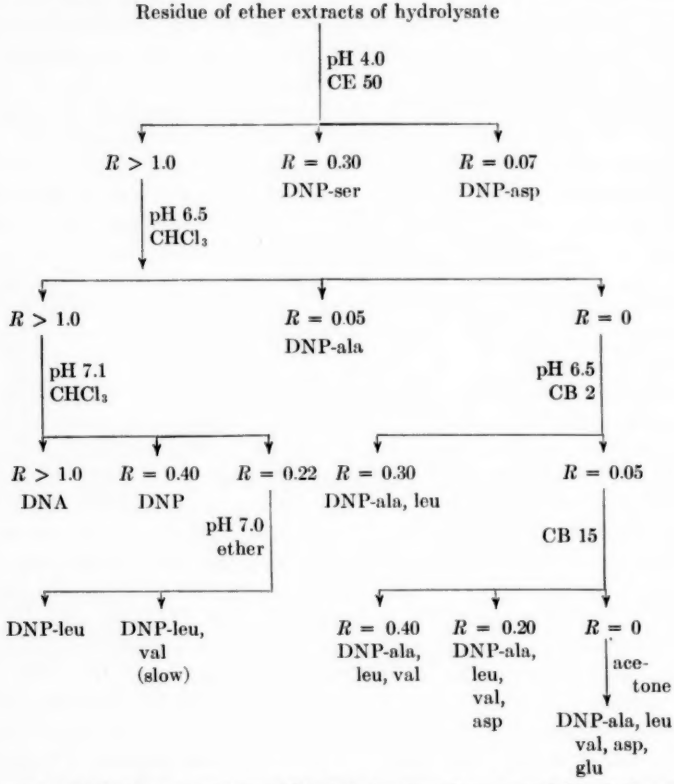
Because the antigen-antibody complex was in some cases treated with DNFB, it was necessary to be certain that DNP derivatives were not formed with the type-specific polysaccharides alone. Such controls were performed with SI, SIII, and SVIII; no colored DNP derivatives could be detected by the procedures used for the study of the DNP proteins.

Hydrolysis of DNP proteins and extraction of DNP derivatives were performed essentially as described by Thompson (12). DNP derivatives were separated by partition chromatography on buffered Celite 545 (13). Columns were prepared immediately before use from a mixture of buffered Celite and a suitable water-saturated solvent, as described by Thompson (12). Phosphate buffers were used at pH 4.0, 5.5, 6.5, and 7.1, citrate buffer at pH 5.0. The following water-saturated solvents were most useful for separation of DNP derivatives of neutral and acidic amino acids on the columns: chloroform, a 1:1 mixture of chloroform and ether (CE 50), 2 ml. of *n*-butanol to 98 ml. of chloroform (CB 2), 15 ml. of *n*-butanol to 85 ml. of chloroform (CB 15), ethyl acetate, and ether. Separation of ε-DNP-lysine was accomplished on a 1 N HCl column with CB 15. The method of Bailey (14) was used for identification of derivatives of other basic amino acids.

The residues of ether extracts of the hydrolyzed DNP proteins were dissolved in a minimal amount of CE 50 and transferred quantitatively to the surface of a CE 50 column at pH 4.0. Development of the yellow band with CE 50 separates DNP-aspartic acid (R = 0.07), DNP-serine (R = 0.23), DNP-glutamic acid (R = 0.35), and DNP-threonine (R = 0.6); all other compounds travel more rapidly ($R > 1.0$) on this solvent-column system. (R is defined as the ratio of the distance traveled by the band in the column to the distance moved by the solvent above the column.) If a good separation of DNP-serine and DNP-glutamic acid was not obtained on this column, the mixed band was resolved with ethyl acetate on a pH 5.5 column (DNP-glutamic acid, R = 0.10; DNP-serine, R = 0.35). The fast band from the column at pH 4.0 then was taken to dryness, dissolved in the minimal amount of chloroform, and separated with chloroform on a column of pH 6.5. On this column, DNP-alanine and DNP-proline (R = 0.02), DNP-valine and DNP-methionine (R = 0.25), and DNP-phenylalanine and di-DNP-lysine (R = 0.56) can be separated from DNP-leucine, DNP-isoleucine, dinitrophenol, and dinitro-

aniline ($R > 1.0$). DNP-glycine and di-DNP-tyrosine, which do not move ($R = 0$), can be eluted from this column with ether, ethyl acetate, or acetone. The fast band from the column at pH 6.5 can be chromatographed with chloroform on a column at pH 7.1 which will separate DNP-leucine and isoleucine ($R = 0.15$ to 0.25) from dinitrophenol ($R = 0.45$) and dinitroaniline ($R = 1.0$). Porter (11) has described other solvent systems which will separate pairs of DNP amino acids with the same R values if both are present in the hydrolysate.

Isolated DNP derivatives were estimated quantitatively with a Beckman spectrophotometer. HCl (1 N) was used for ϵ-DNP-lysine at 390 mμ. Extinction coefficients which were determined for all derivatives encountered were in agreement with those recently reported by Rao and Sober



SCHEME 1. Method of separation of DNP derivatives from a partial hydrolysate of rabbit antibody XIV. DNA, dinitroaniline; DNP, dinitrophenol; ser, serine, asp, aspartic acid, ala, alanine, leu, leucine, val, valine, glu, glutamic acid.

(15). After quantitative estimation, each band was chromatographed on paper for positive identification with one or more solvent systems; *e.g.*, tertiary amyl alcohol (16), 1.5 M phosphate buffer (17), and toluene-pyridine-ethylene chlorohydrin (18). Recovery values determined by Sanger and Porter (19) were used in calculations.

Isolation of DNP Peptides—The same buffered columns and solvents which were used to separate DNP amino acids were employed for the peptide derivatives (for example, see accompanying Scheme 1). Each eluted band from the partial hydrolysates was dissolved in 1 per cent $NaHCO_3$, and the absorbancy at 350 mμ was determined. An arbitrary value, 1.56×10^4, for the extinction coefficient of all DNP peptide solutions was used. The bicarbonate solution was acidified and the DNP derivative extracted into ethyl acetate. The extracts were washed twice with water and taken to dryness *in vacuo*. The residue of DNP peptide was dissolved in 3 to 6 drops of 6 N HCl with warming, sealed in a capillary tube, and heated at 105° for 20 hours. The cooled hydrolysate was transferred to a small tube and extracted with ether. The ether extracts were chromatographed on paper for the identification of the DNP derivative as described above. The aqueous phase was evaporated to dryness, freed of excess acid, and chromatographed on Whatman No. 1 paper with the following solvent systems: butanol-acetic acid (20), tertiary amyl alcohol (21), or phenol-water-ammonia (22). Dry chromatograms were sprayed with the ninhydrin reagent of Levy and Chung (23) and slowly warmed, while still wet, until the spots developed. The distinctive colors of different amino acids produced by this reagent were useful for identification of the compounds.

Free Amino Groups of Antibodies

For the most part, the proteins were treated with DNFB for 3 hours. Inasmuch as some *N*-terminal amino acids have been found to be liberated during this treatment (24), the mother liquors of the DNP proteins were examined for DNP amino acids. Several DNP amino acids (aspartic acid, alanine, valine, leucine or isoleucine, serine, and threonine) were found to be present after this treatment, and additional trace amounts of DNP amino acids (asparagine and glutamic acid) were liberated after treatment with DNFB for 16 hours. None of these was present in an amount greater than 0.05 mole per 160,000 gm. of protein. Presumably these amino acids result from partial breakdown of the protein.

In Table III are the results for all of our studies on rabbit antibodies and γ-globulins. In addition to DNP-alanine and ε-DNP-lysine, significant amounts of DNP-aspartic acid were found in all cases. Besides these DNP derivatives, trace amounts of DNP-valine, DNP-leucine, DNP-serine, DNP-threonine, and DNP-glutamic acid were isolated from most

of the preparations. No DNP derivatives of glycine, proline, arginine, or histidine (as the di-DNP derivative) could be detected by the special methods used for finding them (11).

Because it is known that some breakdown of the protein occurs during treatment with DNFB (24), the presence of trace amounts of DNP amino

TABLE III

Free Amino Groups of Rabbit γ-Globulins and Antibodies

Preparation	Moles per 160,000 gm. of protein			Lysine per 100 gm. of protein	Lysine residues per 100 gm. of protein
	Alanine	Aspartic acid	Lysine		
				gm.	*gm.*
γ-Globulin I	1.17	Present	72	6.61	5.79
" "	1.18	0.46	77	7.05	6.20
" Ib*	0.99	0.41	66	6.02	5.30
" "	1.04	0.48	69	6.30	5.52
" M	0.87	0.36	70	6.36	5.56
" "	1.30	0.41	69	6.30	5.52
" N	1.04	0.29	73	6.65	5.87
" "	0.93	0.28	68	6.20	5.40
Antibody I	0.99	0.41	68	6.24	5.47
" II	0.96	0.47	73	6.66	5.82
" "	0.95	0.38	76	6.82	5.95
" III	0.96	0.36	68	6.18	5.40
" IIIa†	1.14	0.52	70	6.36	5.56
" IV	1.08	0.48	68	6.17	5.40
" V	0.87	0.35	72	6.58	5.77
" "	0.90	0.50	69	6.25	5.50
" VII	0.89	0.42	68	6.20	5.42
" VIII	1.17	0.36	67	6.08	5.35
" XIV	1.04	0.33	70	6.35	5.56
Average	1.03	0.40	70	6.39	5.60
" deviation	±0.10	±0.06	±2	±0.22	±0.19

* This is a different preparation from γ-globulin I.

† The antibody was precipitated by SIII in the presence of 3 moles of aspartic acid.

acids is not surprising. The presence of relatively large amounts of DNP-aspartic acid, however, is somewhat unexpected. Free aspartic acid was recovered from the DNP compound (25) and identified by its position and characteristic color with ninhydrin on a paper chromatogram. In order to test whether free aspartic acid was adsorbed on the antibody, a preparation of type III antibody was precipitated by SIII in the presence of 3 equivalents of aspartic acid. This antibody preparation (IIIa), which was treated in the usual manner, did not yield any significant increase in DNP-aspartic acid. Therefore, the presence of this compound cannot be attrib-

uted to adsorption. It is more likely that there is a labile linkage in the molecule, *e.g.* serylaspartyl or threonylaspartyl, which is hydrolyzed to some extent during the treatment with DNFB, resulting in the formation of DNP-aspartic acid. The possibility that the DNP-aspartic acid originates from a peptide or protein impurity seems very unlikely in view of its almost constant recovery from the various types of antibody and from different batches of γ-globulins. It is conceivable that some of the antibody and γ-globulin molecules (approximately 40 per cent) may be branched polypeptide chains with one *N*-terminal alanine and one *N*-terminal aspartic acid. Such a possibility cannot be excluded but, if this is the case, the branched molecules are present in surprisingly constant amounts in the different samples.

Porter (3) did not observe any DNP-aspartic acid in the hydrolysates of DNP-antiovalbumin or normal γ-globulin. He used unbuffered columns of silica gel and mixtures of chloroform and butanol for separation of DNP derivatives; under these conditions DNP-aspartic acid does not move on the column. Porter reported the presence of a stationary band, but concluded that it was an artifact, since he was unable to identify it as any DNP amino acid.

Regardless of the origin of the DNP-aspartic acid, it is evident from the data in Table III that all of the antibody and γ-globulin preparations yield the same quantity of DNP-alanine and ε-DNP-lysine. These results are identical with those previously obtained by Porter (3), who found an average of 71 lysine residues and 1 *N*-terminal alanine in antiovalbumin and normal rabbit γ-globulin. The average recovery of ε-DNP-lysine is equivalent to 5.60 gm. of lysine residues per 100 gm. of protein, and is in excellent agreement with the average figure, 5.66 gm., obtained by chromatography of antibody hydrolysates on Dowex 50 (4). The recovery of 1 mole of DNP-alanine per 160,000 gm. of protein is in accord with the conclusion of Porter that these proteins consist of a single polypeptide chain with *N*-terminal alanine.

N-Terminal Sequence of Rabbit Antibodies

The method of separation, the approximate *R* values, and the identification of the DNP compounds isolated from partial hydrolysates of four rabbit antibodies are shown in Scheme 1 for one example, antibody XIV. Although some variations of these procedures were used for the other antibodies, Scheme 1 illustrates the general approach. All of the DNP amino acids and peptides which were isolated, and the approximate yield of each compound, are given in Table IV. No correction factors for breakdown of the DNP derivatives were used in calculation of the yields. After hydrolysis of each DNP peptide, the DNP amino acid and the free amino acids were identified as described above.

TABLE IV

DNP Amino Acids and Peptides Isolated from Partial Hydrolysates of Rabbit Antibodies

Amino acids given in parentheses were identified after hydrolysis of the DNP peptide.

Antibody No.	Hydrolytic conditions	Compounds identified	Yield per 160,000 gm. of protein	Yield of DNP-alanine and its peptides
			mole	*mole*
I	6 N HCl, 1 hr., 110°	DNP-aspartic acid	0.25	0.86
		DNP-glutamic acid	Trace	
		DNP-threonine	"	
		DNP-serine	0.09	
		DNP-leucine	0.05	
		" (valine)	0.06	
		DNP-alanine	0.33	
		" (leucine)	0.22	
		" (" valine)	0.23	
		" (" " aspartic acid)	0.08	
VII	12 N HCl, 6 days, 37°	DNP-aspartic acid	0.11	**0.73**
		DNP-serine	0.03	
		DNP-leucine	0.04	
		" (valine)	0.02	
		DNP-alanine	0.49	
		" (leucine)	0.15	
		" (" valine)	0.04	
		" (" " aspartic acid)	0.03	
		" (" " " " glutamic acid)	0.02	
VIII	12 N HCl, 6 days, 37°	DNP-aspartic acid	0.20	**0.57**
		DNP-serine	0.05	
		DNP-glutamic acid	0.05	
		DNP-threonine	0.03	
		DNP-leucine	0.03	
		DNP-alanine	0.33	
		" (leucine)	0.15	
		" (" valine)	0.05	
		" (" " aspartic acid)	0.02	
		" (" " " " glutamic acid)	0.02	
XIV	12 N HCl, 6 days, 37°	DNP-aspartic acid	0.17	0.87
		DNP-serine	0.15	
		DNP-leucine	0.06	
		" (valine)	0.05	
		DNP-alanine	0.31	
		" (leucine)	0.15	
		" (" valine)	0.17	
		" (" " aspartic acid)	0.17	
		" (" " " " glutamic acid)	0.07	

It is evident that the DNP-alanine peptides which were isolated from three of the antibodies are consistent with the sequence, alanylleucylvalyl-aspartylglutamyl, which was also found by Porter to occur in normal γ-globulin and antiovalbumin. Porter (3) identified glutamyl as the fifth residue only tentatively; in our work decisive evidence was obtained repeatedly for this residue in the 5 position. No pentapeptide was isolated from the partial hydrolysate of DNP antibody I. This particular hydrolysis was performed in 6 N HCl rather than 12 N HCl, a condition which would presumably facilitate cleavage of the aspartylglutamyl bond.

The occurrence of DNP-leucylvaline in three of the four hydrolysates is noteworthy. It has been mentioned above that small amounts of DNP-leucine and DNP-valine are present in complete hydrolysates of the antibodies. This finding and the occurrence of DNP-leucylvaline suggest that rabbit antibodies exist which lack the *N*-terminal alanine or alanylleucine residues or, alternatively, that these bonds at the *N*-terminal sequence are labile and are hydrolyzed during the treatment with DNFB. The presence of DNP-aspartic acid and DNP-glutamic acid might also be explained by an unusual lability of the *N*-terminal sequence.

DISCUSSION

The work presented in this paper and the earlier study of Porter (3) leave little doubt that normal rabbit γ-globulin and a variety of antibodies produced in this species all possess a single polypeptide chain with *N*-terminal alanine and with the same number of free ε-amino groups of lysine. In addition, it has been found that the *N*-terminal pentapeptide sequence, alanylleucylvalylaspartylglutamyl, occurs in the normal globulin and the antibodies of this species. The significance of these observations for theories of antibody formation has already been noted by Porter (3), and will be discussed further in the accompanying paper (4).

It should be emphasized that the present results apply only to rabbit antibodies. Evidence already has been presented that the γ-globulins of the human (26, 27), the cow, and the horse (26) are mixtures of proteins with a variety of end-groups. Further studies on the γ-globulins of these species will be presented later.[1]

SUMMARY

1. For eight specific pneumococcus antibodies and normal rabbit γ-globulin it has been found that there is an average of 1 mole of *N*-terminal alanine and 70 ε-amino groups of lysine which will react with 1,2,4-fluorodinitrobenzene (DNFB). In addition, approximately 0.4 mole of aspartic acid and traces of other amino acids are obtained as the dinitrophenyl

[1] We would like to acknowledge the advice and criticism of Dr. E. O. P. Thompson and Adrienne R. Thompson in these studies.

(DNP) derivatives from complete hydrolysates of the DNP proteins. It is likely that these originate from partial breakdown of the protein, mainly at the *N*-terminal end, during the treatment with DNFB.

2. For antibodies of types VII, VIII, and XIV, the *N*-terminal pentapeptide sequence, alanylleucylvalylaspartylglutamyl, has been identified positively, and for type I antibody, the corresponding alanyl tetrapeptide. This *N*-terminal sequence is identical with that found earlier by Porter for rabbit antiovalbumin and normal rabbit γ-globulin. In addition small amounts of DNP-leucylvaline were isolated; this peptide could originate from antibody deficient in *N*-terminal alanine or produced by the treatment with DNFB.

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AMINO ACID COMPOSITION OF FOUR RABBIT ANTIBODIES*

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Only a few incomplete analyses have been reported for the amino acid composition of rabbit antibodies (1). In addition to the value of characterizing such proteins, it is of some interest to determine whether antibodies of different immunological specificity possess the same or a different composition. Such findings have an immediate and obvious bearing on current theories of antibody formation (2–4).

In the present paper we wish to report studies of the composition of four different antipneumococcal antibodies obtained from rabbit serum. These studies were performed on materials precipitated by the specific polysaccharides. The present results suggest that there are no differences sufficiently great to be detected by the methods used; *i.e.*, chromatographic separation of the amino acids on Dowex 50 and analysis by the ninhydrin method. This finding, in conjunction with the observations that rabbit antibodies of different immunological specificity possess identical free amino groups and *N*-terminal pentapeptide sequence (5, 6), strongly suggests that these proteins possess an identical polypeptide sequence. Such a view is in accord with the theories of antibody formation which assume that antibodies differ only in specific surface configuration (2, 3).

Materials and Methods

The materials analyzed were the antibodies to types I, VII, VIII, and XIV pneumococci obtained by precipitation with the specific polysaccharides. The antigen-antibody complex was washed and dried in the manner already given (5). Hydrolysis was performed in 3 times glass-distilled 6 N HCl under anaerobic conditions, and the hydrolysates were worked up by the procedures previously described (7, 8). The amount of protein hydrolyzed was determined on aliquots by micro-Kjeldahl analyses on the basis that the N content of the antibodies is 16 per cent. Determinations by the Dumas method on two normal rabbit γ-globulin preparations gave an average value of 16.0 per cent for the anhydrous, ash-free protein.

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Chromatography of the hydrolysates was performed on Dowex 50-X8 by the methods of Moore and Stein (9). A 0.9 × 100 cm. column was used for acidic and neutral amino acids and a 0.9 × 15 cm. column for the basic amino acids. The eluates were collected in 1 ml. fractions with an automatic fraction collector and the amino acids estimated by the ninhydrin method (10).

Additional samples of each protein were oxidized with performic acid by the method of Schram *et al.* (11) before hydrolysis. The hydrolysates were chromatographed on a 0.9 × 15 cm. column of Dowex 50-X8 and cysteic acid, which emerged at the column volume, was estimated by the ninhydrin method (10).

Tryptophan was determined by the method of Spies and Chambers (12). Because of the insolubility of the antigen-antibody complexes, the following procedure was used. Samples (5 mg.) were suspended in 10 ml. of *p*-dimethylaminobenzaldehyde solution (300 mg. in 100 ml. of 19 N H_2SO_4) and the mixtures kept in darkness until complete solution occurred, usually in 12 to 18 hours. Color was developed by the addition of 0.1 ml. of 0.04 per cent $NaNO_3$. Maximal color was achieved after 30 minutes in darkness. The absorbancy was determined with a Coleman junior spectrophotometer. It was found that the antibodies possess a maximal absorption at 630 mμ, whereas tryptophan itself and many proteins yield a maximum at 600 mμ. In order to circumvent this difficulty, a preparation of human γ-globulin II-1,2 was used as the standard. This protein was found to contain 2.62 ± 0.05 per cent tryptophan, as determined by the method of Bates (13), and is in accord with the value reported earlier by Smith, Greene, and Bartner (14).

Because of the presence of the polysaccharide antigens in the precipitated complexes, the carbohydrate content of the antibodies was not determined. Estimations were made on a non-immune electrophoretically homogeneous preparation of rabbit γ-globulin. Hexose was determined by the orcinol method of Pirie (15) with glucose as the standard; absorbancy was estimated at 420 mμ. Hexosamine was determined by the procedure of Palmer *et al.* (16) with glucosamine as the standard; readings were made at 530 mμ. The Coleman junior spectrophotometer was used for these determinations.

Results

Since certain amino acids are progressively destroyed (*e.g.*, serine and threonine) and others are slowly liberated (*e.g.*, valine and isoleucine) under the conditions of hydrolysis (7, 8, 17), all the materials were hydrolyzed for 20 and 70 hours. Fig. 1 shows a typical run for type VII antibody and Fig. 2 for type XIV antibody, each after 70 hours hydrolysis.

The individual data, averages, and average deviations for the four antibodies studied are given in Tables I and II. It is evident that progressive

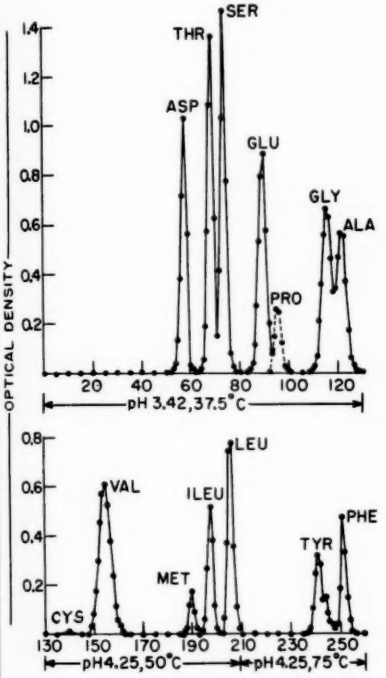

FIG. 1

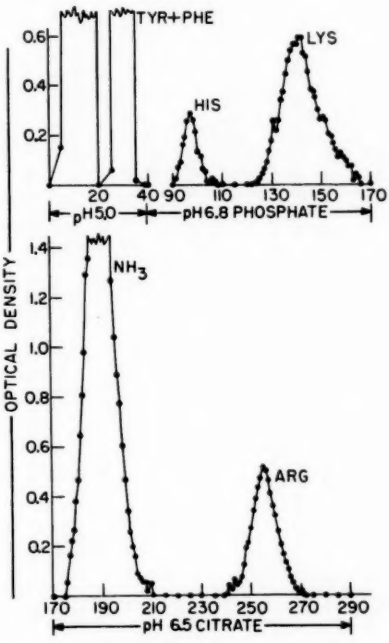

FIG. 2

FIG. 1. Elution curves for a 70 hour hydrolysate of type VII antibody (1.81 mg.) on a 0.9 × 100 cm. column of Dowex 50. The ninhydrin color yields have been corrected for base-line color but not for the different color yields of the amino acids. The color produced by proline was read at 440 mμ, and that produced by the other substances at 570 mμ. ASP, aspartic acid; THR, threonine; SER, serine; GLU, glutamic acid; PRO, proline; GLY, glycine; ALA, alanine; CYS, cystine; VAL, valine; MET, methionine; ILEU, isoleucine; LEU, leucine; TYR, tyrosine; PHE, phenylalanine.

FIG. 2. Elution curves for a 70 hour hydrolysate of type XIV antibody (8.80 mg.) on a 0.9 × 15 cm. column of Dowex 50. The base-line color has been subtracted but no correction has been applied for different color yields of the eluted substances. HIS, histidine; LYS, lysine; ARG, arginine.

decomposition of serine and threonine occurs and, as a result, the ammonia recovery increases with time of hydrolysis. Estimations of these substances were made by extrapolation to zero time. Inasmuch as the antibodies themselves contain some polysaccharide, and each preparation con-

TABLE I

Amino Acid Recoveries from Hydrolysates of Type I and Type VII Antibodies

Data are given as gm. of amino acid residue per 100 gm. of protein. Values in parentheses are omitted from the averages. Values cited as averages for serine, threonine, and ammonia were obtained by extrapolation to zero hydrolysis time.

Amino acid residue	Type I					Type VII				
	Time of hydrolysis				Average	Time of hydrolysis				Average
	20 hrs.		70 hrs.			20 hrs.		70 hrs.		
Aspartic acid	8.53	8.76	8.40	8.80	8.62 ± 0.13*	7.78	8.13	8.31	8.30	8.13 ± 0.18*
Threonine	11.44	10.78	9.23	9.26	11.86 ± 0.17	9.99	10.70	10.03	10.40	10.40 ± 0.27
Serine	8.70	7.86	6.50	6.11	9.07 ± 0.31	8.52	8.61	7.98	7.80	8.84 ± 0.07
Glutamic acid	9.77	9.82	10.56	11.00	10.29 ± 0.49	10.48	10.04	10.12	10.11	10.19 ± 0.15
Proline	8.18	6.71	7.09	6.59	7.14 ± 0.52	7.51	6.56	6.98	7.30	7.09 ± 0.32
Glycine	4.79		4.26	4.56	4.54 ± 0.18	4.51	4.24	4.28	4.24	4.32 ± 0.10
Alanine	4.78		4.43	4.74	4.65 ± 0.15	4.77	4.76	4.21	4.55	4.57 ± 0.19
Valine	(8.01)		8.35	8.45	8.40 ± 0.05	(7.56)	(7.42)	8.20	8.54	8.37 ± 0.17
Methionine	1.59		1.18	1.06	1.28 ± 0.21	1.31	1.26	1.35	1.28	1.30 ± 0.03
Isoleucine	(4.08)		4.47	4.02	4.25 ± 0.23	(3.19)	(3.31)	3.59	3.77	3.68 ± 0.09
Leucine	7.02		7.38	6.85	7.08 ± 0.19	6.62	6.31	6.40	6.88	6.55 ± 0.20
Tyrosine	6.48		5.61	6.03	6.04 ± 0.29	5.67	6.04	5.98	5.97	5.92 ± 0.12
Phenylalanine	4.25		4.94	4.91	4.70 ± 0.30	4.39	4.51	4.70	4.48	4.52 ± 0.09
Histidine	1.31	1.40	1.28		1.33 ± 0.05	1.29	1.26	1.16	1.18	1.32 ± 0.02
Lysine	5.69	5.68	5.59		5.65 ± 0.03	5.70	5.55	5.74	5.87	5.72 ± 0.09
Ammonia	(1.81)	1.53	2.40		1.21	1.43	1.45	1.91	1.95	1.24 ± 0.02
Arginine	4.66	4.48	4.59		4.58 ± 0.06	(3.91)	4.32	4.37	4.45	4.38 ± 0.05
Tryptophan†					2.47 ± 0.09					2.43 ± 0.00
Half cystine†					2.72 ± 0.12					2.79 ± 0.19

* Average deviation.

† Estimated separately.

TABLE II

* Average deviation.
† Estimated separately.

TABLE II

Amino Acid Recoveries from Hydrolysates of Type VIII and Type XIV Antibodies

Data are given as gm. of amino acid residue per 100 gm. of protein. Values in parentheses are omitted from the averages. Values cited as averages for serine, threonine, and ammonia were obtained by extrapolation to zero hydrolysis time.

Amino acid residue	Type VIII					Type XIV				
	Time of hydrolysis				Average	Time of hydrolysis				Average
	20 hrs.		70 hrs.			20 hrs.		70 hrs.		
Aspartic acid	8.28	8.12	8.51	8.33	8.31 ± 0.11*	8.30	8.41	8.06	8.68	8.36 ± 0.18*
Threonine	11.22	10.71	9.48	9.58	11.54 ± 0.17	10.22	10.35	9.34	9.13	10.71 ± 0.09
Serine	8.72	8.99	6.70	6.46	9.77 ± 0.13	7.78	8.25	6.67	6.79	8.53 ± 0.17
Glutamic acid	10.20	10.78	10.21	10.70	10.47 ± 0.27	10.56	10.61	10.21	10.51	10.47 ± 0.13
Proline	8.18	7.90	7.31		7.80 ± 0.32		6.49	6.95	7.15	6.86 ± 0.25
Glycine	4.51		4.28	4.27	4.35 ± 0.10	4.37		4.09	4.07	4.18 ± 0.13
Alanine	5.28		4.33	4.04	4.55 ± 0.49	4.04		4.35	4.70	4.36 ± 0.22
Valine	(8.26)		9.10	8.91	9.01 ± 0.10	(8.36)		9.01	8.61	8.81 ± 0.20
Methionine	1.09		1.22	1.25	1.19 ± 0.06	1.04		1.09	1.28	1.14 ± 0.10
Isoleucine	(3.65)		3.66	3.89	3.78 ± 0.12	(3.50)		3.51	3.38	3.45 ± 0.07
Leucine	6.85		6.66	6.98	6.83 ± 0.10	6.72		6.81	6.90	6.81 ± 0.06
Tyrosine	6.09		5.71	5.69	5.83 ± 0.17	6.23		6.10	5.79	6.04 ± 0.17
Phenylalanine	(4.33)		5.20	5.55	5.38 ± 0.18	4.88		4.90	5.10	4.96 ± 0.09
Histidine	1.22	1.50	(1.04)	1.35	1.36 ± 0.10	1.34	1.53	1.28	1.35	1.38 ± 0.08
Lysine	5.50	5.90	5.64	5.69	5.68 ± 0.11	5.31	5.68	5.93	5.50	5.61 ± 0.20
Ammonia	(1.75)	1.55	2.11	2.29	1.25 ± 0.09	1.55	1.69	2.04	2.19	1.42 ± 0.08
Arginine	4.41	4.83	4.66	4.88	4.70 ± 0.16	4.32	4.43	4.45	4.47	4.42 ± 0.05
Tryptophan†					2.43 ± 0.08					2.49 ± 0.01
Half cystine†					2.42 ± 0.03					2.69 ± 0.06

* Average deviation.
† Estimated separately.

tains 4 to 6 per cent of polysaccharide antigen, it may be expected that this material will influence the rate of destruction of various amino acids. Unlike other proteins previously studied in this laboratory (7, 8) by the present methods, the hydrolysates of the antibodies contained some humin.

Methionine may be slowly oxidized to its sulfone, but no estimate for methionine destruction has been made and the results may be low. Methionine sulfone is eluted from Dowex 50 columns with threonine, and the apparent recovery of threonine, especially from 70 hour hydrolysates, may be high. These errors are small, however, compared with the other errors inherent in the method.

As expected from earlier studies (7, 8), recoveries of valine and isoleucine after 20 hours hydrolysis are lower than those obtained after 70 hours; the former values have been omitted from the averages.

The values for cystine are averages of duplicate determinations of cysteic acid but are calculated as half cystine residues. In our hands, control experiments with cystine itself have given recoveries averaging 82 per cent as cysteic acid, whereas Schram *et al.* (11) reported recoveries of about 90 per cent. Because of this uncertainty, no corrections have been applied and the actual recoveries are reported.

Comparison of the average values in Tables I and II indicates that, for most of the amino acids, the differences among the four antibodies are small and within the expected errors of the method; this is evident for aspartic acid, glutamic acid, glycine, alanine, leucine, tyrosine, histidine, lysine, arginine, and tryptophan. For some of the amino acids, the differences are larger, as indicated by the recoveries for serine, threonine, valine, isoleucine, phenylalanine, and cystine. For these amino acids, however, there are uncertainties concerning destruction factors or rate of liberation. Moreover, for most of these amino acids, the differences among the various antibodies are no greater than some individual determinations on the same preparations. We cannot, therefore, regard these differences as indicating a significant variation in composition.

The uncertain degree of destruction of methionine has already been noted. There are greater difficulties in the estimation of proline than of many other amino acids because of its occasionally difficult separation from glutamic acid and low color value with ninhydrin (see also Harfenist (17)).

The average recovery of ϵ-DNP-lysine from various rabbit antibodies and γ-globulins is 5.60 gm. of residue per 100 gm. of protein (5), which is in excellent agreement with the values given in Tables I to III.

A direct estimate of the amide content of a normal γ-globulin gave 1.30 per cent as ammonia or 1.22 per cent calculated as ammonia residues per 100 gm. of protein (5). This result is in good agreement with the deter-

minations on three of the four antibodies studied. The high results on type XIV antibody, and the high values occasionally obtained for ammonia

TABLE III

Average Amino Acid Composition of Rabbit Antibodies

The values are averages obtained from the data in Tables I and II.

Constituent	Residue per 100 gm. protein	Average deviation	Constituent per 100 gm. protein	Nitrogen per 100 gm. protein	Nitrogen
	gm.	*per cent*	*gm.*	*gm.*	*per cent total N*
Aspartic acid	8.36	1.7	9.67	1.02	6.38
Threonine	11.22*	3.1	13.22	1.55	9.69
Serine	9.05*	4.5	10.92	1.46	9.13
Glutamic acid	10.36	1.2	11.80	1.12	7.00
Proline	7.22	4.0	8.56	1.04	6.50
Glycine	4.35	2.3	5.72	1.07	6.69
Alanine	4.53	1.8	5.68	0.89	5.56
Valine	8.65	3.0	10.22	1.22	7.63
Methionine	1.23	4.9	1.40	0.13	0.81
Isoleucine	3.79	6.1	4.39	0.47	2.94
Leucine	6.82	2.1	7.90	0.84	5.25
Tyrosine	5.96	1.3	6.62	0.51	3.19
Phenylalanine	4.89	5.7	5.49	0.47	2.94
Histidine	1.35	1.5	1.53	0.41	2.56
Lysine	5.66	0.7	6.46	1.24	7.75
Ammonia†	1.22‡	0.8	1.30‡	1.07	6.69
Arginine	4.52	2.7	5.04	1.62	10.13
Tryptophan	2.46	0.8	2.70	0.37	2.31
Half cystine	2.66§	4.5	3.13	0.36	2.25
Hexose†	0.93	6.5	1.03		
Hexosamine†	1.13	1.8	1.26	0.10	0.63
Total	105.14		122.74	16.96	106.03

* These average values for serine and threonine were obtained by extrapolating by least squares the sixteen individual determinations. If instead, the four extrapolated values listed in Tables I and II are averaged, the values for gm. of amino acid residue per 100 gm. of protein for threonine and serine are 11.13 and 9.05, respectively. The respective average per cent deviations are 5.1 and 4.1.

† These constituents were determined on whole rabbit γ-globulin and are not averages of determinations on the four antibodies.

‡ These values are omitted from the totals.

§ This is an actual recovery value.

on other antibodies which have been omitted from the calculations, may have been due to accidental contamination by ammonia of the acidic hydrolysates or of the buffers used for elution of the short column.

Composition of Rabbit Antibody

Inasmuch as there do not appear to be significant differences in composition, the combined results of the determinations on each of the four antibodies (Tables I and II) have been averaged and given in Table III. The average deviations are calculated with the average result for each antibody being taken as a single value. These values show that, with the exception of isoleucine and phenylalanine, the deviations are less than 5 per cent from the average and support the view that there is no significant difference in composition of the four antibodies.

TABLE IV

Ionic Groups of Rabbit γ-Globulin

The number of ionic groups is calculated on the basis of a molecular weight of 160,000 and on the assumption that there is a single free α-carboxyl group to conform with the single α-amino group.

Acidic residues	No. of moles per 160,000 gm. protein	Basic residues	No. of moles per 160,000 gm. protein
Aspartic acid	116	Arginine	46
Glutamic "	128	Lysine	71
Terminal carboxyl	1	Histidine	16
		Terminal α-amino	1
Total	245		
Amide groups	−122		
Total anionic groups	123	Total cationic groups	134

The weight and nitrogen recoveries appear to be high, but are within the over-all uncertainties of the methods used. Since the amount of protein in each hydrolysate was estimated on the basis of 16 per cent nitrogen, an error in the nitrogen content will not influence the per cent recovery of this element. Consistently low nitrogen determinations on the hydrolysates would produce high weight and nitrogen recoveries.

The sulfur content of rabbit γ-globulin, calculated from the content of methionine and cystine (the latter corrected for 82 per cent recovery, the average obtained with cystine itself), is 1.32 per cent. This is in reasonable agreement with the value 1.39 per cent obtained by Dr. A. Elek on a sample of normal rabbit γ-globulin.

The partial specific volume of the antibody calculated from the average residue weights in Table III and the specific volumes of the amino acid residues (18) is 0.728.

The number of ionic groups of the antibody has been computed on the basis of 160,000 molecular weight for the protein and on the assumption that there is a single α-carboxyl group to conform with the single α-amino group. The values which are given in Table IV indicate 123 carboxyl groups and 117 lysine plus arginine residues. From these values the isoelectric point should be at a pH value at which six of the imidazole groups are in the cationic form. This might be expected to be nearer neutrality than the isoelectric point at pH 5.8 reported by Tiselius and Kabat (19) for rabbit antibodies.

Comparison of the values in Table III with those reviewed by Smith and Jager (20) for the amino acid content of γ-globulins of other species shows that these proteins all possess a similar composition and that they undoubtedly represent a closely related family of proteins, as noted earlier (14). Particularly noteworthy in all of them is the high content of hydroxyamino acids, of lysine, and, most strikingly, of valine and proline. The content of methionine, histidine, and isoleucine is relatively low.

DISCUSSION

The present investigations have demonstrated that specific rabbit antibodies cannot be distinguished on the basis of amino acid composition or the *N*-terminal pentapeptide sequence. As noted by Porter (6), 19^5 pentapeptides could theoretically occupy the five *N*-terminal positions. It does not seem unreasonable, therefore, to assume that the complete sequence of amino acids in all rabbit γ-globulin molecules is the same. It must be recognized, however, that the sequence of only five residues of approximately 1500 has been established, and that variations of less than 5 per cent in amino acid composition would not be detected with certainty by present methods of amino acid analysis.

The studies of Heidelberger *et al.* (21) and of others (22, 23) on the incorporation of labeled amino acids into antibody and serum proteins demonstrated that serum γ-globulin which is present in the serum is not unfolded and refolded to produce antibody. It seems logical, therefore, to accept the view (3) that normal and immune γ-globulins are formed from the same uncoiled polypeptide precursor and that, in the presence of antigen, the uncoiled chain assumes a folded or coiled structure which is complementary in shape to the antigenic determinant groups. Antibody synthesis, like protein synthesis in general, may be assumed to occur in two phases: first, a process in which amino acids or peptides are combined in a long polypeptide chain and a second phase in which the specific coiling and folding occur to produce the shape of the native protein. Presumably, one may regard the antigen as influencing only the second phase. The observed multiplicity of antibodies to a single antigen can then be due to

the fact that antibodies are complementarily adapted to different portions of the antigen molecule rather than to the production of antibodies of different chemical structure.

The foregoing discussion is, in general, the theory of antibody production suggested by Haurowitz (2) and by Pauling (3). Burnet and Fenner (4) have objected to this theory on the grounds that antibody production is known to continue when antigen can no longer be detected. They have also pointed out that Pauling's theory does not explain the phenomenon of accelerated secondary response to the antigen. These investigators have suggested as an alternative view that the antigen may modify the enzymatic mechanism which produces the antibody. In effect, neither of these objections is insuperable. The fact that antigen cannot be detected in some instances of continued antibody production may be due only to the insensitivity of available techniques. It is possible that cells already producing antibody may be more capable of "trapping" specific antigen and therefore manifest an accelerated response. Whatever the ultimate evidence may turn out to be, both ideas are in consonance with the view that the polypeptide sequence of different antibodies and normal globulin is the same, the different views are concerned only with the manner in which the specific complementary coiling and folding is produced.

Most of the present suggestions concerning the structure of globular proteins assume that the peptide chain exists in some type of coiled or helical structure whose shape is maintained mainly by hydrogen bonding at the peptide bonds. Because the nitrogen of proline in peptide form does not possess a peptide hydrogen, it is unable to form a hydrogen bond and there must, therefore, be a discontinuity of the coiled structure at each proline residue. The protein may be visualized then as bundles of helices or coils connected by proline residues. Large variations in arrangement of the polypeptide coils become possible only if large numbers of proline residues occur in the protein. From this viewpoint, it is worth calling attention to the relatively high proline content of rabbit antibody and of γ-globulins in general (20).

SUMMARY

The amino acid composition of rabbit antibodies to type I, VII, VIII, and XIV pneumococcus has been determined, mainly by chromatography of the hydrolysates on Dowex 50. Within the experimental limitations of the method the composition of the four antibodies is the same. It is suggested that the present results are consistent with theories of antibody formation which assume that antibodies differ in specific surface configuration, whereas the amino acid content and peptide sequence are the same.

XUM

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ACTION OF TRIPEPTIDASE ON DIPEPTIDES OF APPROPRIATE STRUCTURE*

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The specificity of tripeptidase, an enzyme which catalyzes the hydrolysis of tripeptides, *e.g.* glycylglycylglycine, has been studied by Smith *et al.* (1–5) and by Fruton and coworkers (6–8). Although this enzyme exhibits variations in kinetic behavior depending on the species or tissue from which it is derived, the substrate specificity appears to be the same regardless of the source. Tripeptidase is known to hydrolyze only tripeptides containing a free primary or secondary α-amino group, a free terminal α-carboxyl group, and a peptide hydrogen at the sensitive bond. If optically active, the amino acid residue bearing the amino group must be of the L configuration. Ellis and Fruton (8) have further concluded that calf thymus tripeptidase is specifically adapted to act on peptide chains of a particular length characteristic of tripeptides composed of α-amino acid residues.

On the basis of this conclusion, Ziff and Smith (9) have studied the inhibition of tripeptidase from rabbit skeletal muscle and calf thymus, by a group of compounds which bear a structural similarity to a tripeptide. The inhibitors studied included Pyribenzamine, Benadryl, procaine, and pronestyl, all of which possess a chain of 8 to 9 carbon atoms, and which, like tripeptides, have a positively charged group at pH 7.8. All were found to be inhibitory to the action of tripeptidase.

The present paper supports the conclusion of Ellis and Fruton regarding the specificity of the enzyme for substrates of a particular chain length and indicates that "dipeptides" such as glycyl-δ-amino-n-valeric acid and glycyl-p-aminobenzoic acid are also hydrolyzed by equine erythrocyte tripeptidase.

The observation that glycyl-p-aminobenzoic acid but not glycyl-o-aminobenzoic acid is hydrolyzed by an extract of hog intestinal mucosa was made by Balls and Köhler (10). They ascribed the hydrolysis to a dipeptidase present in their "erepsin" preparations. Later, Bergmann *et al.* (11) demonstrated that the action was not due to a "dipeptidase" but to an

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"aminopeptidase." Present studies with a purified enzyme preparation show that the hydrolysis of the para derivative is due to the tripeptidase.

EXPERIMENTAL

Methods—Hydrolysis was measured by the alcohol titration method of Grassmann and Heyde (12). Purified tripeptidase from equine erythrocytes (HD3 (5)) was incubated at 40° with the substrate at 0.05 M concentration in the presence of 0.04 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.8. Inhibitors were used at 0.001 M. 100 per cent hydrolysis represents the scission of one peptide bond. Enzyme activity is expressed as the first order proteolytic coefficient, $C_1 = K_1/E$, where K_1 is the first order rate constant for hydrolysis, calculated in minutes and decimal logarithms. E is the enzyme concentration in mg. of protein N per ml. of the assay solution. The enzyme stock solution was prepared by dissolving a weighed amount of the protein (HD_3) in 8 ml. of water and the protein N concentration was calculated on the basis of a nitrogen content of 16 per cent. The calculated value was also checked by the modified Bücher method (13).

TABLE I

Hydrolysis of Triglycine and Analogues by Erythrocyte Tripeptidase

The assays were made as described in the text. Purified equine erythrocyte tripeptidase, HD_3 (5), was present at 0.024 mg. of protein N per ml. for hydrolysis of triglycine, and at 10 times this concentration for the other substrates.

Substrate	Time	Hydrolysis	K_1	C_1
	hrs.	*per cent*		
Triglycine	0.50	29	0.0050	
	0.75	39	0.0048	
	1.0	48	0.0047	
	1.5	63	0.0048	
Average			0.0048	0.20
Glycyl-δ-amino-*n*-valeric acid	3	15	0.00039	
	5	34	0.00060	
	9	48	0.00053	
	20	73	0.00048	
Average			0.00048	0.0020
Glycyl-*p*-aminobenzoic acid	5	12	0.00020	
	20	27	0.00012	
Average			0.00015	0.00067

Results

The data of Table I show that glycyl-δ-amino-*n*-valeric acid is hydrolyzed at about 1 per cent and glycyl-*p*-aminobenzoic acid at about 0.3 per cent of the rate of triglycine by erythrocyte tripeptidase. The data of Table II show the effect of four inhibitors on these rates of hydrolysis. The same inhibitor decreased the relative activity of the enzyme for each of the three substrates to about the same extent; *i.e.*, cysteine 70 per cent, Benadryl 30 per cent, and Pyribenzamine 20 per cent. Cadmium chloride

TABLE II

Effect of Inhibitors on Erythrocyte Tripeptidase Activity

Assays were performed as described in the text. The concentration of enzyme was 0.024 mg. of protein N per ml. for triglycine and 10 times this level for the other substrates. Inhibitors were used at 0.001 M. Each flask was buffered with 0.04 M Tris buffer at pH 8.0.

Substrate	Inhibitor	C_1	Relative activity
			per cent
Triglycine	None	0.20	100
	$CdCl_2$	0	0
	Cysteine	0.14	70
	Benadryl	0.05	25
	Pyribenzamine	0.03	15
Glycyl-δ-aminovaleric acid	None	0.0020	100
	$CdCl_2$	0	0
	Cysteine	0.0013	65
	Benadryl	0.0007	35
	Pyribenzamine	0.0005	25
Glycyl-*p*-aminobenzoic acid	None	0.0007	100
	$CdCl_2$	0	0
	Cysteine	0.0005	71
	Benadryl	0.0002	28

used at the same concentration (0.001 M) produced complete inhibition in all three cases. These results support the view that the three substrates are being hydrolyzed by the same enzyme.

In addition to the chain length of the substrate, other factors may affect the rate of hydrolysis. In the susceptible compounds there is probably an appreciable difference in the strength of the peptide bond undergoing scission. Also, there is probably a variation in the pK values of the free basic groups of the three substrates which influences the substrate concentration; *i.e.*, the concentration of the ionic species which combines with the enzyme. Since each of these factors will influence the relative rate of hydrolysis of the compounds, explanations of the differences in rate are not justified at present. However, on the basis of an "all-or-nothing"

analysis, there is the feature of "fit" between the enzyme and the substrate as determined by the distance between the amino and carboxyl groups. The data in Tables I and II indicate that the sites of attachment of the substrate on the enzyme can adapt to small variations in the chain length of the substrate as represented in glycyl-δ-amino-*n*-valeric acid and glycyl-*p*-aminobenzoic acid. This is also suggested by the previous work of Smith *et al.* (3, 5) and Fruton and coworkers (7, 8), which showed that glycyl-β-alanylglycine and glycylglycyl-β-alanine are hydrolyzed by tripeptidase. The enzyme has no action on β-alanylglycylglycine; this indicates a rigid requirement that the free amino group must be α to the susceptible peptide bond.

An interesting feature is that glycyl-δ-amino-*n*-valeric acid and glycyl-*p*-aminobenzoic acid are hydrolyzed by tripeptidase, although both are "dipeptides" and do not possess a second peptide bond. This finding does not eliminate the possibility that this grouping is a site of interaction of the substrate and the enzyme, but indicates that such a grouping is of secondary importance.

Preparation of Peptides

Glycyl-*p*-aminobenzoic acid was synthesized by the method of Balls and Köhler (10).

Carbobenzoxyglycyl-δ-amino-n-valeric Acid—An ether solution of carbobenzoxyglycyl chloride (prepared from 12.5 gm. of carbobenzoxy glycine) and 25 ml. of 2 N NaOH were added simultaneously at −5° to a vigorously shaken solution of δ-amino-*n*-valeric acid hydrochloride (7.7 gm.) in 50 ml. of 2 N NaOH. The addition required 20 minutes. Additional 4 N NaOH was added to maintain the mixture alkaline to litmus. The preparation was allowed to come to room temperature and the aqueous phase withdrawn and acidified to Congo red with 6 N HCl. The compound crystallized on chilling and scratching. The product was washed with water and recrystallized from methanol-water. Yield, 7.5 gm.; m.p., 116–117°.

$C_{15}H_{20}O_5N_2$.	Calculated.	C 58.4, H 6.5, N 9.1
308.3	Found.	" 59.0, " 6.6, " 9.0

Glycyl-δ-amino-n-valeric Acid—The carbobenzoxy peptide (4.2 gm.) in methanol was reduced in the usual manner with palladium and hydrogen. The product crystallized during the reduction and was redissolved by addition of hot water. After the catalyst was removed, the product was crystallized by concentrating *in vacuo* and adding ethanol. Yield, 2.0 gm.; m.p., 203–204° with decomposition. It was recrystallized from water-ethanol for analysis.

$C_7H_{14}O_3N_2$.	Calculated.	C 48.3, H 8.1, N 16.1
174.2	Found.	" 48.3, " 8.1, " 16.0

SUMMARY

Equine erythrocyte tripeptidase hydrolyzes glycyl-δ-aminovaleric acid and glycyl-*p*-aminobenzoic acid at 1 per cent and 0.3 per cent, respectively, of the rate of triglycine. These dipeptides can act as substrates of tripeptidase since they possess the required chain length. Inhibition of the enzyme by several compounds indicates that the hydrolysis of these substrates is due to the same enzyme, tripeptidase.

The synthesis of glycyl-δ-aminovaleric acid is described.

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STUDIES ON THE ACTION OF CARBOXYPEPTIDASE ON TOBACCO MOSAIC VIRUS*

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The action of carboxypeptidase on tobacco mosaic virus (TMV) results in the exclusive release of threonine from the virus nucleoprotein (1). The enzyme-treated virus retains full virus infectivity, and its disease symptoms on Turkish tobacco leaves are indistinguishable from those caused by the untreated virus. The treated virus, however, does not reproduce in its modified form, since treatment of the progeny with carboxypeptidase results in the release of the same amount of threonine as one obtains from progeny of untreated virus.

In the present communication additional observations on the nature of the enzyme-virus reaction and on the origin of the liberated threonine are presented, together with additional data on the chemical, physical, and serological properties of the carboxypeptidase-treated virus. The reaction of the enzyme with virus which had been subjected to sonic treatment, and also with detergent-dissociated virus, has also been studied. On the basis of these observations evidence is presented for the occurrence in the molecular structure of TMV of a series of repeating fundamental units.

EXPERIMENTAL

Virus and Enzyme Preparations—Highly purified preparations of TMV were obtained by the standard procedure of differential centrifugation and were stored in aqueous solution in the presence of small amounts of added toluene to prevent bacterial or mold contamination. Supernatant solutions resulting from the sedimentation of the virus from such preparations were found to be free from ninhydrin-reacting substances (see Spot A, Fig. 5).

Carboxypeptidase-treated virus (CT) was obtained by treating TMV with enzyme until the liberation of its available threonine was complete,

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as described below in the section dealing with rate of release of threonine from TMV. The treated virus was isolated from the reaction mixture in the form of a pellet by means of high speed centrifugation, and subsequently redissolved in distilled water containing a drop of toluene as preservative; such a preparation (CT) was used to inject rabbits in order to obtain CT antiserum. In preparing CT for the electrophoretic experiments, the virus was subjected to additional cycles of centrifugation in order to wash out the trace of enzyme occluded in the virus gel. While negligible on a weight basis, this trace of enzyme was sufficient to react with TMV when CT and TMV were mixed and dialyzed for several hours in preparation for certain electrophoresis experiments.

Sonically treated virus, TMV(S), was prepared by subjecting a solution of TMV, containing 20 mg. of virus per ml., to vibrations of 9 kc. for 30 minutes in a magnetostriction oscillator (model S-102A, Raytheon Manufacturing Company, Waltham, Massachusetts). When the virus solution was subsequently centrifuged at 40,000 r.p.m. for 15 minutes in the Spinco model L ultracentrifuge, a small gelatinous pellet was obtained, and the bulk of the material, which remained in the supernatant fluid, was carefully separated from the residual pellet. TMV(S), like the preparation of TMV from which it was derived, was composed of rod-like particles of varying length as shown in Fig. 1. The size of the rods in these treated preparations averaged, however, about one-third that of the intact virus rods. The particles resulting from sonic treatment of TMV show no evidence of chemical alteration and contain the same proportions of protein and nucleic acid as the intact virus (2).

TMV protein, TMV(P), obtained by treating the virus with sodium dodecyl sulfate at pH 8 to 9 and 37° for 24 hours (3), was generously placed at our disposal by Dr. H. Fraenkel-Conrat; the absence of *N*-terminal groups in the same preparations has already been reported (4). The preparation was completely free from virus nucleic acid and was found according to ultracentrifugal analysis to be composed mainly of material whose molecular weight is 10,000 to 20,000.

The carboxypeptidase preparations used for the enzyme experiments were obtained as crystalline aqueous suspensions from the Worthington Biochemical Sales Company and from the Armour Research Laboratories.[1] Just before use a portion of enzyme was brought into solution by the addition with stirring at 0–4° of 1 per cent $NaHCO_3$, together with a few drops of 0.1 N NaOH; an alternate procedure, which seemed to work well, was simply to add 0.1 N NaOH and to mix at room temperature. The solution was further diluted with distilled water to a concentration of about 0.004 to 0.005 mg. of enzyme N per ml. Crystalline carboxypeptidase may con-

[1] The authors are indebted to Dr. Lawrence L. Lachat of the Biochemical Market Development Section for a generous supply of enzyme.

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tain traces of endopeptidases, but, since these enzymes do not attack native TMV, it was not found necessary to pretreat enzyme solutions with

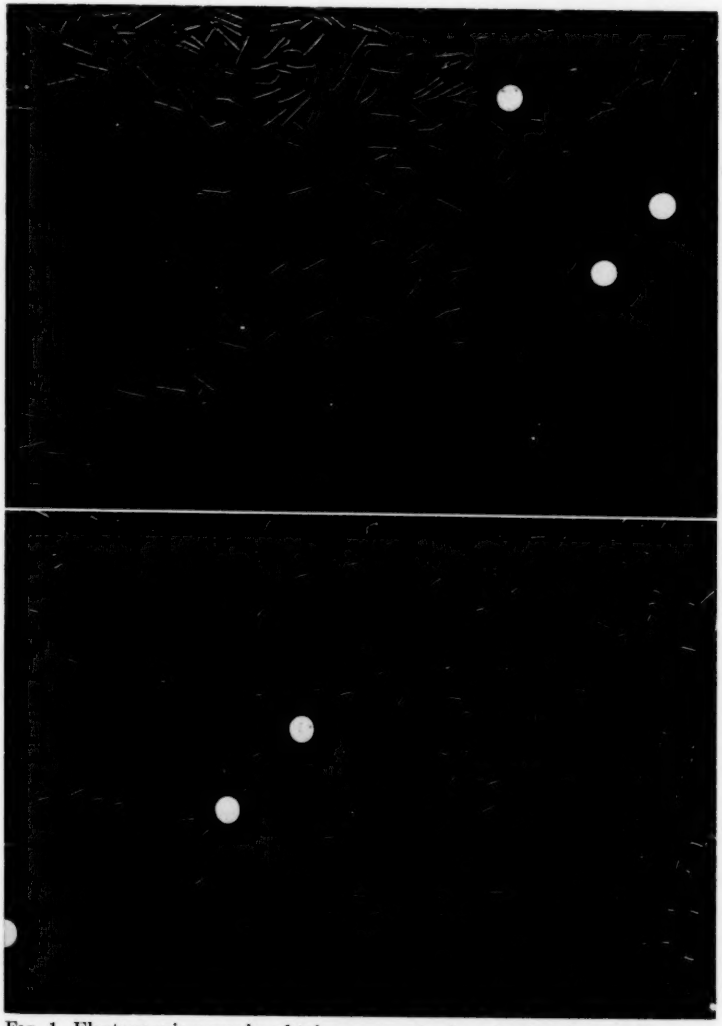

FIG. 1. Electron micrographs of tobacco mosaic virus (top) and sonically treated virus (bottom). The magnification may be calculated by reference to the spherical polystyrene latex particles which are about 260 mμ in diameter.

an endopeptidase inhibitor such as diisopropyl fluorophosphate (DFP); consequently, with TMV and TMV(S) similar results were obtained both

with and without DFP. In the case of TMV(P), however, new end-groups in addition to threonine were found to be released in experiments in which the enzyme had not been treated with a 50-fold excess of DFP prior to its addition to the substrate.

Rate of Release of Threonine from TMV—In a typical experiment an aqueous solution of a fresh, highly purified preparation of the virus (containing 3.2 mg. of virus N per ml.) was incubated with an equal volume of a carboxypeptidase solution (containing 0.005 mg. of enzyme N per ml.)

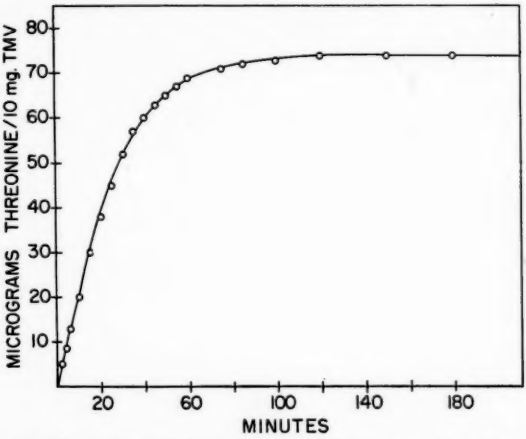

FIG. 2. Rate of liberation of threonine from tobacco mosaic virus upon treatment with carboxypeptidase at pH 7.0 and 25° and at concentrations of enzyme and virus nitrogen of 0.0025 mg. per ml. and 1.6 mg. per ml., respectively. The smooth curve was calculated from an integrated form of the Michaelis-Menten equation (6), and the circles represent experimentally determined points. The values of the constants used in the equation for the curve are $K_m = 1.0 \times 10^{-3}$ mole per liter and $k_3 = 0.022$ mole per liter per minute per mg. of enzyme N per ml.

at pH 7.0 and 25°. Aliquots of the resulting solution were withdrawn at suitable intervals during a digestion period of 3 hours and enzyme action was stopped by addition of 0.1 N HCl to pH 3.0. After removal of the virus by high speed centrifugation, supernatant solutions were collected and their threonine content was estimated by microbiological assay (5). As shown in Fig. 2, the available threonine is completely released during the first 2 hours of reaction, the maximal amount released corresponding to 74 γ of threonine per 10 mg. of virus.

If the amount of threonine released (expressed as per cent reaction) is plotted against the logarithm of time as described by Fraser and Powell (7), the curve shown in Fig. 3 is obtained. When theoretical curves for

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zero and first order reactions are constructed on the same scale, the rate of release of threonine from TMV appears to follow zero order kinetics in the initial stages and later to become first order in the region of low substrate concentration. Release of threonine from the virus is thus shown to follow the course which might be predicted from the classical Michaelis-Menten theory for an enzyme-catalyzed reaction at constant enzyme concentration (8).

A direct demonstration of conformity of the TMV-carboxypeptidase reaction to the Michaelis-Menten theory is afforded by application of an integrated form of the Michaelis-Menten equation (6) as follows:

$$k_3 e t = 2.3 K_m \log \frac{a_0}{a} + (a_0 - a) \qquad (1)$$

As pointed out by Elkins-Kaufman and Neurath (6), Equation 1 is a composite first order and zero order expression. When data from the TMV-carboxypeptidase reaction are substituted in this equation, which is then solved for the constants, and the equation is plotted, the smooth curve shown in Fig. 2 is obtained. Application to this plot of experimentally determined values for the threonine released from TMV after various reaction times results in the fit shown in Fig. 2. The excellence of fit thus shown can be considered strong evidence for adherence of the kinetics to the Michaelis-Menten theory. The value for K_m, 1.0×10^{-3} mole per liter, is approximately 10 times that found for synthetic model substrates (9).

On the basis of the above considerations, an interpretation of the virus-enzyme reaction can be made as follows. In the early stages of the reaction, the effective concentration of the substrate is high; thus, on the assumption that each mole of virus nucleoprotein contains approximately 2900 moles of a reactive subunit, a substrate-enzyme mole ratio of approximately 1000:1 will apply. Consequently, in the initial stages of the reaction the rate of formation of the virus-enzyme complex is likely to be considerably faster than its rate of dissociation and the rate of release of threonine; in this event the second right-hand term in Equation 1 would predominate; hence the rate of release of threonine from the virus would be expected to approach zero order kinetics.

If, however, as the concentration of TMV diminishes, the rate of formation of the virus-enzyme complex becomes considerably slower than the rate of its decomposition, enzyme-substrate complex formation becomes the rate-determining step; the first right-hand term of Equation 1 will now predominate and as a result release of threonine from the virus should become a first order reaction at low concentrations of TMV.

As shown in Fig. 3, these expectations are fulfilled. The rate of release of threonine from the virus approximates zero order kinetics in the initial

stages of the reaction and subsequently approximates a first order reaction as the concentration of substrate becomes small.

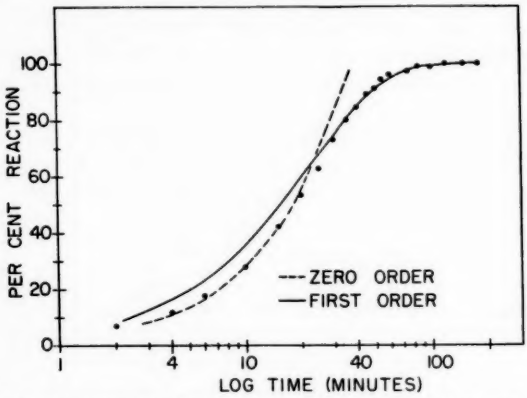

FIG. 3. Rate of release of threonine from TMV expressed as per cent reaction against logarithm of time.

TABLE I

Effect of Added Threonine on Reaction between Tobacco Mosaic Virus and Carboxypeptidase

Reaction time	Threonine released per 10 mg. virus*	
	TMV control	TMV + 70 γ threonine
min.	γ	γ
1	5	2
5	20	20
10	38	37
20	56	56
30	66	67

* The reactions were run at pH 7 and 25° and at concentrations of 1.6 mg. of virus N and 0.0025 mg. of enzyme N per ml. To the reaction mixture represented by the last column were also added 70 γ of threonine per 1.6 mg. of virus N. The threonine was determined by the ninhydrin method (see the text).

An alternative explanation for the course followed by the carboxypeptidase-TMV reaction is that the threonine released during the reaction begins to exert an inhibitory effect in the later stages. However, in an experiment which is summarized in Table I, this did not seem to be the case, for the addition of threonine in an amount approximately equivalent to the maximum expected had virtually no effect on the general course of the reaction.

Quantitative Estimation of Threonine—As indicated above, many of the threonine determinations were made by means of microbiological assay (5). Some determinations were also made by the quantitative ninhydrin method of Moore and Stein (10) with threonine as a standard. Finally, in order to obtain an accurate and independent check of the amount of threonine released in the TMV-carboxypeptidase reaction, the dinitrophenyl (DNP) procedure (11) was applied to a supernatant solution obtained as described above.

A 1 ml. aliquot of the supernatant solution from a 3 hour digest of TMV was allowed to react with dinitrofluorobenzene (DNFB) at pH 9.0 and 40° for 90 minutes; excess reagent was removed by extracting the reaction solution with ether. The aqueous solution was then acidified to pH 1.0 with HCl and again extracted with ether to remove the DNP derivative (or derivatives) of the products of the carboxypeptidase reaction. The combined ether extracts were concentrated *in vacuo*, and the dinitrophenol formed by hydrolysis of DNFB was removed by sublimation; the residue was then examined by two-dimensional chromatography on paper (11). As expected, only one spot, corresponding to DNP threonine, was obtained. The DNP amino acid was eluted with 1 per cent $NaHCO_3$ (4 ml.) and the optical density of the solution at 360 mμ was measured in a Beckman spectrophotometer.

In a separate experiment the above procedure was applied to a known amount (1 μmole) of pure DL-threonine, and the optical density readings of such standard solutions were used to calculate the amount of threonine (in micromoles) present in supernatant solutions obtained from carboxypeptidase-TMV reaction mixtures. This procedure automatically compensates for experimental losses, and furthermore an accurate value for the molecular extinction coefficient of DNP threonine need not be known.

In this way it was estimated that 69.2 ($\pm$2) γ of threonine are released from 10 mg. of TMV by the action of carboxypeptidase; this value is in good agreement with that of 70 $\pm$ 4 γ obtained by microbiological assay on the same solution and is equivalent to 1 threonine residue per unit of molecular weight of 17,200 on the basis of a molecular weight of about 50×10^6 for TMV (12).

Action of Carboxypeptidase on TMV(S) and TMV(P)—In order to obtain further evidence concerning the possible existence of a chemical subunit in TMV a study was made of the action of carboxypeptidase on dissociated fragments obtained from TMV by sonic treatment and by the action of sodium dodecyl sulfate as described earlier; these procedures as applied here are presumed not to break covalent bonds.

Solutions containing TMV, TMV(S), and TMV(P) (containing the equivalent of 20 mg. of virus per ml.) were each mixed with equal volumes of a carboxypeptidase solution (containing 0.064 mg. of enzyme per ml.,

which had been pretreated with a 50-fold excess of DFP at room temperature). The TMV and TMV(S) reaction solutions were maintained at pH 7.0, but the TMV(P) solution was allowed to react at pH 8.0 in order to keep the substrate in solution during the course of the enzyme reaction. Aliquots of the respective reaction solutions were withdrawn at suitable intervals during a 4 hour digestion period and enzyme action in each aliquot was terminated by the addition of 0.1 N HCl to pH 3.0. TMV and TMV(S) were removed by high speed centrifugation and the threonine content of the supernatant solutions was estimated by microbiological assay. Although TMV(P) did not form a pellet in the ultracentrifuge,

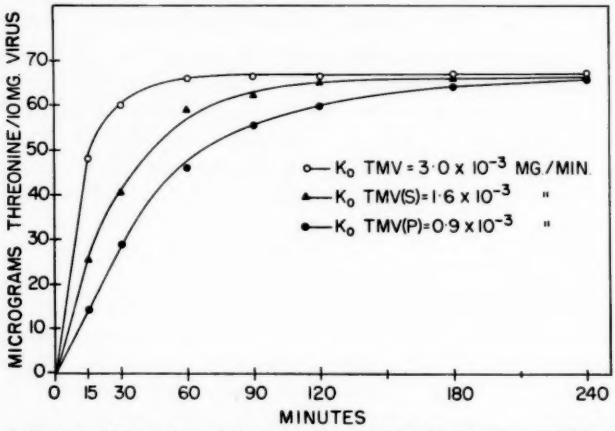

FIG. 4. Rates of liberation of threonine from TMV, TMV(S), and TMV(P). The reactions were run at 22° and at pH 7 except for TMV(P), which was held at pH 8. The reaction mixtures contained 10 mg. of substrate and 0.032 mg. of enzyme per ml. and the enzyme was pretreated with a 50-fold excess of diisopropyl fluorophosphate.

assay for threonine was successfully performed in the presence of the protein substrate. The results showing the rates of release of threonine from the three substrates are presented in Fig. 4.

Release of threonine from TMV was complete after 1 hour and, as expected, amounted to 67 (± 3) γ from 10 mg. of virus. From the sonically treated virus, threonine was released at a slower rate, but after about 2 hours reached a maximal value of 66 (± 3) γ from 10 mg. of TMV(S). In the case of TMV(P) the reaction rate was still slower despite the fact that the reaction was carried out at a pH which is considered nearer the optimum for the action of the enzyme. Thus, a maximum corresponding to 67 $\pm$ 3 γ of threonine from 10 mg. of substrate was reached with TMV(P) only after 4 hours of digestion.

For paper chromatographic studies aliquots of the supernatant solutions

taken after 3 hours digestion, and corresponding to about 5 mg. of virus, were freed from residual protein by the "molecular sieve" procedure (13, 14). Thus after high speed centrifugation the supernatant solutions were shaken mechanically for 1 hour with Amberlite IR-120 resin in the H^+ form; the protein solutions were removed by means of a pipette, and the

FIG. 5. Chromatogram obtained with supernatant solutions from carboxypeptidase-treated virus preparations with butanol-acetic acid-water, 4:1:5, as the developing solvent and Whatman No. 1 paper. Spot A, TMV control, without enzyme; Spot B, TMV-carboxypeptidase; Spot C, threonine; Spot D, TMV(P)-carboxypeptidase; Spot E, TMV(S)-carboxypeptidase.

residual resin was washed several times with distilled water to remove last traces of protein. The resin was then eluted with 5 N NH_4OH and aliquots of the ammonia eluates were chromatographed on Whatman No. 1 paper with butanol-acetic acid-water (4:1:5) as the developing solvent. As shown in Fig. 5, threonine exclusively is released from all three substrates, indicating that no new *C*-terminal groups are revealed in the virus molecule after sonic or detergent treatment.

Effect of Elevated Temperature on Carboxypeptidase-TMV Reaction—In

order to test the possibility that more threonine or that other amino acids might be released from TMV by carboxypeptidase at an elevated temperature, duplicate reactions were run at 23° and 37°. The relative concentrations of enzyme and virus and other conditions were those previously described.

Paper chromatography of the supernatant fluids from the reaction mixtures showed threonine to be the only amino acid present in significant amount in either case. Quantitative analysis of the threonine, made by the ninhydrin method of Moore and Stein (10), gave identical values for the supernatant fluids at 23° and 37°, namely, the equivalent of 66 γ of threonine per 10 mg. of virus.

In other experiments made in this laboratory,[2] elevated temperature, 10 times the concentration of enzyme, and several times the reaction period were employed, still without appreciably changing the outcome.

These results further strengthen the conclusion that threonine is the *only* product of the reaction of carboxypeptidase on TMV and that the maximal amount of threonine is released under the mild conditions used in most of our experiments.

Influence of Host on Nature of C-Terminal Groups of TMV—In order to investigate the possibility that the threonine released from TMV by the action of carboxypeptidase might represent an unusual type of impurity associated with the culture of the virus in Turkish tobacco, highly purified preparations of TMV were obtained from Turkish and white Burley tobacco and from phlox plants. Each preparation was treated with carboxypeptidase at pH 7 to 7.5 for 2 hours at room temperature. After removal of the virus by high speed centrifugation, the supernatant fluids were assayed for threonine in the usual manner. By using two separate preparations of each, values of 65 and 61, 61 and 58, and 61 and 63 γ of threonine[3] per 10 mg. of virus were obtained for TMV from Turkish tobacco, white Burley tobacco, and phlox, respectively. From these results it can be concluded that the *C*-terminal threonine associated with TMV is characteristic of the virus and not of the host from which the TMV is obtained.

Effect of Endopeptidases on Carboxypeptidase-Treated TMV—Although it is well known that trypsin and chymotrypsin do not attack native TMV, it seemed of interest to investigate whether the virus becomes more susceptible to proteolytic degradation after its initial reaction with carboxypeptidase.

Virus which had undergone complete reaction with carboxypeptidase

[2] Dr. H. Fraenkel-Conrat, personal communication.

[3] These results are slightly low, presumably owing to decreased activity of the enzyme preparation used, since other experimental evidence also indicated this to be the case.

was adjusted to a pH of 7.5 and treated with crystalline trypsin and chymotrypsin, respectively. Each reaction mixture contained 10 mg. of CT and 0.01 mg. of enzyme, and the reaction was allowed to proceed at room temperature (22°) for 6 hours. CT was removed from the reaction solutions by centrifugation at 40,000 r.p.m. for 1 hour and the supernatant fluids were withdrawn, concentrated, and chromatographed on paper with butanol-acetic acid-H_2O (4:1:5) as the developing solvent. No ninhydrin-reacting material was detected; hence it is concluded that CT, like native TMV, is not susceptible to hydrolysis by trypsin and chymotrypsin.

Physicochemical Properties of Carboxypeptidase-Treated TMV—Preparations of TMV and CT were kindly examined in the electron microscope by Dr. R. C. Williams using the spray droplet technique in preparation of the mounts (15). The electron micrographs obtained showed nothing distinctive in the morphology of the CT particles, since the bulk of the material was represented by rod-like particles about 300 mμ in length with shorter and longer rods each present in approximately the same numbers commonly observed in purified preparations of TMV.

The sedimentation behavior and electrochemical properties of TMV and CT in 0.1 M cacodylate buffer at pH 6.8 were kindly investigated by Dr. H. K. Schachman, using the Spinco model E centrifuge and the Perkin-Elmer and Spinco electrophoresis apparatus.

Both the ultracentrifuge patterns and the sedimentation coefficients were in agreement with the results of electron microscopy in indicating that treatment of TMV with carboxypeptidase effects no substantial change in the size, shape, or density of the virus particles. The individual preparations of TMV and CT exhibited single boundaries in the electrophoresis apparatus in a sodium cacodylate-cacodylic acid buffer at an ionic strength of 0.1 and pH 6.9. Although the electrophoretic mobilities of TMV and CT were similar, -7.4×10^{-5} and -7.9×10^{-5} cm. per second per volt per cm. for TMV and CT, respectively, resolution was obtained, as illustrated in Fig. 6, when the two were studied in a mixture. These results show that treatment of TMV with carboxypeptidase leads to a material with greater electrophoretic mobility at pH 6.9.

Serological Properties of Carboxypeptidase-Treated TMV—Antisera to TMV and CT were obtained by subjecting rabbits to a course of eight spaced intravenous injections. A total of 58 mg. of each substance was injected into the appropriate rabbit. About 5 days after the last injection, the rabbits were bled by cardiac puncture and the sera were separated from the clotted blood. Four rabbits were employed, two for each antigen.

Strong reactions and cross-reactions were observed in precipitin tests with the various sera and antigens, thus demonstrating strong serological relationships. In order to detect possible qualitative differences in the antigens, TMV and CT, as reflected in their antisera, cross-absorption ex-

periments were performed under conditions similar to those previously described (16, 17).

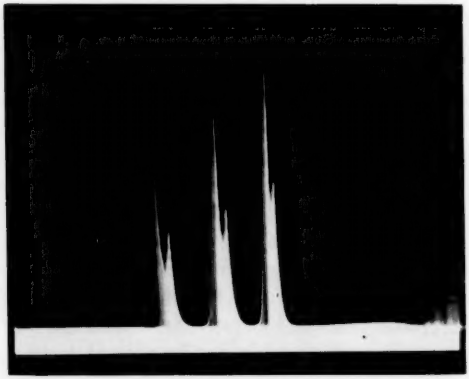

FIG. 6. Electrophoretic patterns of a mixture of tobacco mosaic virus, 1.0 mg. per ml., and carboxypeptidase-treated virus, 1.5 mg. per ml., in a sodium cacodylate-cacodylic acid buffer at ionic strength 0.1 and pH 6.95. The picture is a composite one in which three separate photographs are superimposed. The boundaries are moving to the left and the pictures were taken 140, 185, and 240 minutes, respectively, after the start of the experiment. The field strength was 4.0 volts per cm.

TABLE II

Precipitation of TMV and CT with Absorbed Antisera

Antiserum absorbed	Antigen used for absorption	Precipitin tests with absorbed sera	
		Antigen	
		TMV	CT
TMV	CT	+++*	−
CT	TMV	−	+++

* +, degree of precipitation at the optimum; −, no precipitation. The precipitin tests were made at five different concentrations of antigen ranging from 0.006 to 0.1 mg. in 0.1 ml. to which 0.1 ml. of antiserum was added.

As shown in Table II, the cross-absorption tests clearly indicate a significant difference between the antigenic properties of TMV and CT, for, after maximal removal of antibodies by the heterologous antigen, a specific precipitate was still obtained in the homologous reaction.

Effect of Dilute Acid and Alkali on TMV—A possibility arising from the apparent absence of any significant number of *N*-terminal groups in TMV

(4) is that TMV is made up of cyclic peptide chains and that the threonine residues removed by carboxypeptidase do not really represent *C*-terminal groups, but rather are side chain residues which are linked in the virus either singly or as polythreonine chains to γ-carboxyl groups of glutamic acid residues. To test these possibilities, the stability of TMV at pH 2.8 and 11 was investigated.[4]

An aqueous solution of TMV (2 ml., containing 10 mg. of virus per ml.) was brought to pH 2.8 with 0.1 N HCl and kept at 100° for 1 hour. The coagulated virus was centrifuged at 20,000 r.p.m. for 1 hour, and the supernatant solution was separated from the pellet of denatured virus.

A similar solution of TMV was brought to pH 11 with 0.1 N NaOH and heated at 100° for 1 hour. The solution remained clear and no pellet was recovered after centrifugation at 20,000 r.p.m. for 1 hour; in order to separate degradation products of low molecular weight the solution was subjected to the "molecular sieve" resin procedure already described.

The supernatant solution at pH 2.8 and the material adsorbed by the resin from the solution at pH 11 were chromatographed on Whatman No. 1 paper in butanol-acetic acid-water (4:1:5). By this means, aspartic acid was found to be the only amino acid released from the virus at pH 2.8 and 100°,[5] while at pH 11 small amounts of free serine, glycine, and threonine could be detected. Their combined amount, however, represents less than 5 per cent of the threonine which would be released from an equivalent amount of virus under the action of carboxypeptidase. In view of these results, the presence of polythreonine units or of γ-glutamyl peptide linkages in TMV is considered to be unlikely

DISCUSSION

In our preliminary communication (1) it was reported that approximately 80 γ of threonine were released from 10 mg. of TMV by the action of carboxypeptidase. However, more extensive studies, also involving reevaluation of threonine standards, have yielded a revised value reported herein of 69 (±2) γ of threonine per 10 mg. of virus. On this basis it may be calculated that 2900 (±100) residues of threonine are released per mole of TMV, assuming a molecular weight of 50,000,000 for the virus.

By analogy with the action of carboxypeptidase on synthetic substrates of known chemical structure (9), and in view of its successful application to the *C*-terminal group analysis of several protein substrates (21), it is

[4] These conditions were chosen because γ-peptide linkages are known to be more labile to acid than normal α-peptide bonds (18) and because it would be predicted that polythreonine would behave similarly to polyserine which has been shown to be particularly labile in alkaline solution (19).

[5] The release of aspartic acid upon treatment with dilute acid has also been noted with other proteins (20).

reasonable to assume that the threonine residues released from the virus represent the *C*-terminal amino acids of a corresponding number of polypeptide chains in the virus molecule.[6] Accordingly, it may be postulated that each constituent peptide chain represents a chemical subunit, of molecular weight 17,000, within the virus molecule. If a fundamental subunit is calculated on the basis of 1 cysteine residue per peptide chain[7] of the virus, a value of approximately 18,000 is derived for its molecular weight. The presence of such a repeating subunit in the virus molecule is also consistent with the results of x-ray diffraction studies (22, 23), which indicate a great degree of regularity in the virus structure and the presence of regular repeat units.

Although the precise molecular size of the protein moiety obtained by treating TMV with sodium dodecyl sulfate is not known, the material appears to behave like a protein of low molecular weight (3); in fact, preliminary data from this laboratory indicate that it has a molecular weight in the range of 10,000 to 20,000 (4). A molecule of this size is compatible with the calculated subunit proposed above, and, further, the fact that threonine is released from such a protein preparation in an amount which corresponds to 1 mole of threonine per unit of molecular weight 17,000 provides strong support for the concept that the virus molecule is composed of a series of repeating fundamental units.

Additional support for the subunit is provided by the kinetics of the virus-carboxypeptidase reaction, which by conformity to the Michaelis-Menten theory indicate a uniformity of the threonine released and hence imply an origin from identical subunits.

The fact that TMV is dissociated by treatment with sonic vibrations and by detergents indicates that the structural units of TMV are probably held together by relatively weak bonds such as hydrogen bridges. This view is strengthened by the present findings because no new *C*-terminal groups appear to be created by sonic and detergent treatments of the virus,[8] and in all cases essentially the same quantity of threonine is released per unit weight of virus.

The nature of the amino acid residues which occur adjacent to *C*-terminal threonine in the virus molecule has not been established, but it appears that the residual structure resulting from the enzymatic cleavage of threonine does not meet the conditions required for the continued action

[6] It is possible that the reaction observed with TMV is not due to carboxypeptidase but to a contaminating enzyme not susceptible to inhibition by diisopropyl fluorophosphate. However, the few anomalous reactions reported with carboxypeptidase so far (21) have proved to be inhibited by DFP, and we feel that the idea of a contaminant is a rather unlikely explanation of the pronounced reaction observed with TMV.

[7] Cysteine is the amino acid which occurs in least amount in TMV (5).

[8] Likewise, no *N*-terminal groups appear to be produced in detergent-treated virus (4).

of carboxypeptidase. Some of the specificity requirements of this enzyme have been established by studies on synthetic substrates (9), but it has become apparent from recent studies on proteins and polypeptides (21, 24, 25) that the specificity of carboxypeptidase is dependent not only on the nature of the amino acid sequences but also on over-all environmental conditions. Nevertheless, by analogy with the B chain of insulin, in which alanine is the only amino acid released from the terminal sequence ... Pro.Lys.Ala, an analogous sequence (*viz.* ...Pro.Lys.Thr) can be postulated to explain the specific release of threonine from TMV.[9]

The observed changes in serological specificity and in electrophoretic mobility of the carboxypeptidase-treated virus both indicate a significant alteration of the surface chemistry of the virus, and it seems likely that determination of the actual nature of the penultimate residues in TMV will contribute something to the understanding of both electrophoresis and serology.

The present structural impasse with TMV illustrates the limitations of the carboxypeptidase method for the elucidation of amino acid sequences from the carboxyl end of polypeptide chains. An unequivocal answer to this question must await the development of other methods applicable to stepwise degradation from the carboxyl end.

Finally, in considering the peptide chains of TMV, it is apparent that the *C*-terminal end is only half the picture. The *N*-terminal groups of TMV and its dissociated protein moiety have been sought by the DNFB and phenylisothiocyanate procedures (4). No appreciable amounts of such groups, however, were found in any of the preparations studied; furthermore, the ϵ-amino groups of the lysine residues in the virus appear to account for its total amino nitrogen content when estimated by the Van Slyke procedure (26). In view of these findings it was postulated (4) that the α-NH_2 groups of the *N*-terminal residues in the virus molecule may be masked by combination with the ω-carboxyl groups of aspartic and glutamic acid residues, thus forming 6-shaped peptide chains containing *C*-terminal threonine. A similar hemicyclic structure has been proposed for tropomyosin by Bailey (27).

The authors are indebted to Dr. Arthur B. Pardee for helpful suggestions concerning the kinetic aspects of this investigation.

SUMMARY

The action of carboxypeptidase on tobacco mosaic virus (TMV), sonically treated TMV, and detergent-treated TMV has been investigated.

[9] Evidence has recently been obtained by Dr. Niu and Dr. Fraenkel-Conrat of this laboratory, using a hydrazinolysis technique, that the actual sequence is ... Pro.Ala.Thr.

In all cases, threonine exclusively is released as a result of the enzymatic action. The maximal amount of threonine released is found to be the same for all three types of virus preparations, although the rates of release are different. The reaction with TMV is also shown to conform to the Michaelis-Menten theory for an enzyme-catalyzed reaction at constant enzyme concentration.

The biological properties, and the size, shape, and density of the carboxypeptidase-treated virus are found to be indistinguishable from those of the untreated virus. However, the electrophoretic mobility and the serological properties of the treated virus are demonstrably different.

From quantitative determination it is calculated that 2900 ($\pm$100) threonine residues are released from TMV by the action of carboxypeptidase, and it is concluded that these represent the *C*-terminal groups of an equal number of peptide chains in the virus structure. From these, and other considerations, it is concluded that TMV is comprised of about 2900 subunits, each having a molecular weight of approximately 17,000.

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THE ACTION OF CARBOXYPEPTIDASE ON STRAINS OF TOBACCO MOSAIC VIRUS*

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In studies on the chemistry of virus mutation, thirteen strains of tobacco mosaic virus (TMV) have been characterized with respect to their protein and nucleic acid constituents (1–4). A possible chemical basis for virus mutation was provided by the finding that mutant strains of TMV can differ in amino acid content, whereas they seem not to differ in the composition of their nucleic acids. However, no chemical differences were found between some strains, and, while this may be attributable to a need for refining the analytical tools, it is also possible that these strains differ only in certain structural features not reflected in their compositions.

In this connection, it was recently found (5, 6) that the enzyme, carboxypeptidase, releases specifically from TMV about 2900 residues of threonine per mole of virus, from which it was concluded that threonine is the primary carboxyl terminal (*C*-terminal) residue of the native virus and, by combination with other data, that the virus is made up of about 2900 peptide chains representing subunits of the virus having a molecular weight of about 17,000. As indicated above, it was noted that carboxypeptidase does not digest along the peptide chains of TMV, but stops sharply after removal of threonine. Since these results appear to distinguish TMV from most proteins tested thus far (7), it seemed desirable to examine for such structural features all of the strains of TMV which had previously been analyzed for protein and nucleic acid components. The results of this investigation, together with preliminary data obtained on other plant viruses, are the subject of this report.

EXPERIMENTAL

Virus Preparations—Highly purified preparations of the various strains of TMV and other plant viruses were obtained from appropriate plants by the customary procedure of differential centrifugation (1, 4, 8, 9). Aqueous solutions of such preparations were used in the reaction with carboxypeptidase.

Consistent results were obtained only with strictly fresh virus prep-

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arations, for older preparations tended to develop bacterial growth, which, even though slight, frequently led upon treatment with carboxypeptidase to release of amino acids which were not observed with freshly prepared virus. This effect could not always be abolished by repurifying the virus, *e.g.* by differential centrifugation. This suggests the possibility that some bacteria may possess enzymes capable of altering the structure of TMV and other viruses, although it is also possible that autolysis of bacteria could release protein material which had sedimentation characteristics similar to those of the virus and which was susceptible to the action of carboxypeptidase. In any case, erratic results were frequently obtained when old preparations of the viruses were treated with carboxypeptidase; hence fresh, highly purified preparations were employed in the major part of these studies, although, in a few instances, older preparations which had been preserved with toluene were used.

Reaction with Carboxypeptidase—The conditions for the enzyme reaction were in most cases similar to those previously described (5, 6); *i.e.*, the reaction was carried out at about pH 7.5 and approximately 25° for 2 to 8 hours with about 1.6 mg. of virus N and 0.004 mg. of enzyme N per ml. The carboxypeptidase used was an aqueous suspension, crystallized three times, obtained from the Worthington Biochemical Sales Company, Freehold, New Jersey. In some instances the reactions were run after incubation of the enzyme with an inhibitor such as diisopropyl fluorophosphate or tetraethyl pyrophosphate (10) to preclude activity of traces of endopeptidases. With strains of TMV and most of the plant viruses except potato virus X, this precaution was unnecessary, owing to the well known resistance of these viruses to trypsin and chymotrypsin. At the end of the reaction period, the viruses were removed from the reaction mixtures by centrifuging at 40,000 r.p.m. for 1 hour in the Spinco model L ultracentrifuge. The supernatant fluids were carefully drawn off down to the level of the virus pellets and analyzed.

Analyses of Digestion Products—Qualitative analyses of the reaction mixtures after removal of substrate were made by means of paper chromatography following an approximate 60-fold concentration of aliquots of the supernatant fluids. Concentration was achieved either by lyophilization or by evaporation to dryness in a stream of nitrogen. In either case the residue was dissolved in a small volume of water and 1 to 20 μl. aliquots were applied to Whatman No. 1 paper for chromatography in phenol-water or in butanol-acetic acid as previously described (5, 11).

On the basis of the results of the qualitative tests for amino acids, quantitative analyses were made either by microbiological assay (4) or by application of the photometric ninhydrin method of Moore and Stein (12).

Tests for Biological Activity of Carboxypeptidase-Treated Viruses—The

carboxypeptidase-treated viruses were removed from the enzymatic digestion mixtures by high speed centrifugation and after resuspension were tested on appropriate plant hosts by rubbing a leaf or two with dilutions containing approximately 10^{-5} gm. of virus per ml. The symptoms developing in the inoculated plants were noted.

TABLE I

Maximal Yields of Threonine Obtained from Strains of Tobacco Mosaic Virus by Reaction with Carboxypeptidase at 25° (Approximately 1 Part of Enzyme N Per 400 Parts of Virus N)

Virus strain	Threonine, γ per 10 mg. virus
TMV	65*
M	66
J14D1	60
GA	64
YA	70
HR	70
B2	65
B2A	63
B3	72
B4	67
S1	61
S2	67
S3	66

* The values are averages of two or more determinations made in most cases by the quantitative ninhydrin method, although for TMV approximately equal numbers of analyses were made by this means and by microbiological assay. In the case of TMV twenty-three determinations were made on nine different preparations of virus and the standard deviation calculated was 5.

Results

Qualitatively and quantitatively, tobacco mosaic virus and twelve of its strains reacted with carboxypeptidase in essentially the same manner. Threonine and only threonine was detected in the enzymatic digests in all cases. An average of 65 γ of threonine per 10 mg. of virus was released from TMV, and the average values obtained for twelve strains of TMV, as indicated in Table I, were very similar in magnitude.

As illustrated in Table II, the behavior of other plant viruses when treated with carboxypeptidase differed in two major respects from TMV and its strains: (1) several different amino acids were evidently released as a consequence of treatment with the enzyme (it was not determined which of these represented primary *C*-terminal groups and which adjacent residues) and (2) the sum of all the amino acids released for a given weight of

virus was considerably smaller in all cases than the quantity of threonine obtained from a comparable weight of TMV.

The differences between cucumber viruses 3 and 4 and the tobacco mosaic group were especially striking in view of the common consideration that these cucumber viruses are strains of TMV. The primary *C*-terminal group of the cucumber viruses appears to be alanine rather than threonine, for about 80 per cent of the amino acid released in a 6 hour treatment with carboxypeptidase was found to be alanine. This point was established from a combination of the paper chromatography results, the data from

TABLE II

Reaction of Some Plant Viruses with Carboxypeptidase

Virus	Amino acid released, γ per 10 mg. virus*	Amino acids identified as reaction products by paper chromatography
Tobacco mosaic	65	*Threonine*†
Potato X	9	*Alanine*, aspartic acid, glutamic acid, leucine,‡ threonine, valine
Southern bean mosaic	3	*Alanine*, leucine, valine
Tomato bushy stunt	7	*Valine*, *leucine*, alanine
Tobacco ringspot	3	*Serine* and possibly arginine
Cucumber 3 and 4	5	*Alanine*, leucine, phenylalanine, valine

* Determined as amino nitrogen by the quantitative ninhydrin reaction and expressed in terms of threonine. The reactions were run at about pH 7.5 and at 25° for 6 hours with about 1.6 mg. of virus N and 0.004 mg. of enzyme N per ml., the enzyme having been previously treated with tetraethyl pyrophosphate.

† The amino acids identified as major spots are italicized.

‡ Or isoleucine in all cases in which leucine is listed.

amino nitrogen determinations, and the figures obtained in microbiological assays for alanine.

As judged by the symptoms produced on the usual hosts, none of the viruses was substantially affected by treatment with carboxypeptidase, for the symptoms produced in each case upon inoculation with suitable dilutions of the treated virus were indistinguishable from those obtained with untreated virus.

DISCUSSION

It might have been supposed from the diverse protein compositions of some of the strains of TMV (1, 4) that these viruses would differ in their peptide structures, and particularly in the nature of their *C*-terminal residues. However, this does not seem to be the case, for all thirteen strains examined appear to have only threonine as the *C*-terminal residue and,

furthermore, probably have similar structures adjacent to the threonine in view of the fact that carboxypeptidase action on these strains stops in every case after release of the threonine.

While it would not be justified to conclude that the occurrence of *C*-terminal threonine is a unique feature of TMV and its strains, it is clear that this structural feature distinguishes the TMV family from the few other plant viruses tested thus far. In fact, generalizing from the present data it appears that it can be considered a chemical indication of strain relationship when viruses are found to contain the same numbers and type of *C*-terminal residues. (Another chemical marker of strain relationship recently proposed is that strains of a virus will have the same quantity of nucleic acid and that their nucleic acid compositions will be identical (13).)

In addition to the close similarity of TMV strains with respect to *C*-terminal groups, it should be pointed out that other fundamental structural inferences can be drawn. In the case of TMV, evidence has been adduced to support the idea of a fundamental subunit in the virus structure (6, 13). The existence of this unit and its size have been deduced from x-ray studies, content of the limiting amino acid, cysteine, and the number of *C*-terminal residues. Calculations from these data indicate the presence of a subunit with a molecular weight of approximately 17,000.[1] Actual chemical subunits obtained by treating the virus with hydrogen bond-breaking reagents are of a size consistent with the calculated unit (14). Admittedly, the present data do not permit the conclusion that all strains of TMV contain precisely the same number of *C*-terminal residues; nevertheless, it is clear that the strains examined (Table I) contain very similar if not identical numbers of such residues. Therefore, it can be postulated that strains of TMV contain the same number of chemical subunits of approximately the same size, and that this is a characteristic structural feature of virus strains. Support for this hypothesis is provided by the observation that all strains of TMV examined contain the same quantity of cysteine, which, on the assumption of 1 cysteine residue per subunit, leads to a calculated subunit which is close in size and number to that computed from the *C*-terminal data. These structural similarities seem especially significant when the strain, HR, is compared with TMV, for these two strains are grossly different in proportions of most of their constituent amino acids.

The *C*-terminal analyses reported for plant viruses other than the TMV

[1] This value was calculated from the maximal amount of threonine released by carboxypeptidase from a standard amount of virus in a carefully conducted kinetic experiment following which the threonine was determined by three independent types of analysis (6). If the average value given for TMV in Table I is used, the calculated subunit comes out about 18,000, but, since the data in Table I represent a heterogeneous set of conditions and less rigorously controlled experiments, the value given above is considered a better estimate.

group must be considered only indicative of what may be found in more detailed studies with varied conditions, because the present experiments on these viruses were conducted solely on a comparative basis with TMV and its strains. If higher enzyme concentrations, higher temperatures, and longer reaction times are employed, the picture may change for the other plant viruses, although the situation for TMV is virtually unaffected by such variations in conditions (6). In any case, under the present moderate conditions, evaluation of the carboxypeptidase reaction for the non-TMV viruses was rendered difficult by the multiplicity of the products of the enzymatic action and their low concentrations.

Incidentally, the character of the carboxypeptidase results indicates that the *C*-terminal groups of plant viruses are not vitally concerned *per se* in their capacity to infect, since infectious quality was unimpaired by treatment of the strains with the enzyme. Furthermore, it might be inferred that the nature of the *C*-terminal groups has little or no effect on the type of symptoms induced in the host, since removal of the primary *C*-terminal groups from the TMV strains and from other plant viruses has no apparent effect on the symptoms caused in the usual host. However, so little virus gets into the host cells to initiate infection that the pathological effects are likely due directly or indirectly to the progeny, and, in the case of TMV, it will be recalled that the progeny of carboxypeptidase-treated virus were found to have the *C*-terminal characteristics of the untreated virus (5). Another way of summarizing these results is to say that the hereditary centers of these viruses are apparently unaffected by treatment with carboxypeptidase.

SUMMARY

Each of thirteen distinctive strains of tobacco mosaic virus was treated with carboxypeptidase and it was found that essentially the same quantity of threonine, and nothing but threonine, was split from each as a result of the enzymatic treatment. In contrast, smaller quantities of several amino acids were split from potato X, southern bean mosaic, tomato bushy stunt, and tobacco ringspot viruses and from cucumber viruses 3 and 4. From the results obtained it appeared that the number and type of carboxyl-terminal residues were characteristic of a given virus and hence were the same for all strains of that virus. Since strains of tobacco mosaic virus appear to contain the same number of carboxyl-terminal residues, namely about 2900, it was concluded that they are also probably composed of the same number of chemical subunits.

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AN ADAPTATION OF THE FLOYD-LAVINE PROCEDURE FOR THE ISOLATION OF METHIONINE TO TRACER WORK*

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(Received for publication, October 21, 1954)

Floyd and Lavine (1) reported a procedure for the isolation of methionine from protein digests as the methionine methylsulfonium bromide. The method was based on the observation (2) that methionine, on digestion in 16 to 18 N H_2SO_4 in the presence of methanol, is quantitatively converted to methionine methylsulfonium salt. Before applying the procedure to the isolation of methionine from tissues of animals which were given various radioactive precursors of methionine, it appeared desirable to establish that the methyl group of methionine and of methionine methylsulfonium salt is not subject to exchange with the methyl of methanol when the latter is present in excess in the sulfuric acid digestion mixture. It was also deemed necessary to ascertain that the methionine methylsulfonium salt, isolated from animal tissues, is chromatographically pure. In the course of this investigation it was observed that cystine, cystathionine, cysteine, and homocystine, on digestion with sulfuric acid in the presence of methanol, form a compound or compounds which are precipitable with phosphotungstic acid together with the methionine methylsulfonium salt formed from methionine at the same time. However, the phosphotungstates obtained from cystine, cysteine, cystathionine, and homocystine are separated from the phosphotungstate of methionine methylsulfonium by virtue of a much greater solubility of the former in ethanol. Homocysteine does not form a thiolactone in strong sulfuric acid (3), and hence, in the presence of methanol, it will be methylated to methionine methylsulfonium salt. However, as far as we know, homocysteine has not been detected in animal proteins, and the interference by homocysteine with methionine isolation from protein digests does not present a serious problem. Homocysteine thiolactone readily forms phosphotungstate salt, but the latter, if present, is removed in the first treatment of the deacidified protein digest with phosphotungstic acid.

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The principle of the procedure described below is that of Floyd and Lavine (1). It differs from that described by these authors in manipulative details which are essential for the successful isolation of the sulfonium salt from animal protein digests in pure form and in good yields, and for this reason it is described here in full. We are indebted to Dr. T. F. Lavine and Mr. N. F. Floyd for making their procedure available to us before its publication. An account of the present work has been presented (4).

EXPERIMENTAL

Preparation of Animal "Proteins"—Intact rats, mice, or chicks, or the organs of these animals were used. After the hides and feathers were removed, the entire animal (or the organs) was ground up and extracted with boiling ethanol, followed by ethyl ether. The alcohol-ether extracts were set aside for the isolation of phospholipides, choline, creatine, and cholesterol. The lipide-free material was extracted with 10 per cent NaCl solution at 85° for 6 hours to remove the nucleic acids. The saline-extracted material was extracted with several portions of 6 per cent trichloroacetic acid at room temperature, washed with water, then with absolute ethanol, followed by ether, and dried. The material is referred to as "proteins."

Isolation of Methionine Methylsulfonium Bromide from "Proteins"—1 to 10 gm. of the "proteins" was digested with 30 volumes of 6 N HCl for 6 hours, the digest was evaporated *in vacuo* to a syrup, and as much HCl as possible was removed by distillation with water. The residue was dissolved in water (5 ml. per gm. of protein) and decolorized with three fresh portions of activated carboraffin. The light yellow filtrate was warmed on a water bath, and a hot solution of phosphotungstic acid in water was added (3 gm. of phosphotungstic acid in 5 ml. of water per gm. of protein). The mixture, after heating on a steam bath for 30 minutes, was kept in the dark overnight. The precipitated phosphotungstates were removed by filtration and washed with 75 ml. of a solution of 2 gm. of phosphotungstic acid containing 2 ml. of 1 N HCl. The filtrate and washings (purplish) were treated with 1 M tetraethylammonium bromide (about 0.2 ml. per gm. of protein) to remove the excess of phosphotungstic acid. More of the tetraethylammonium bromide is added, if necessary, until the filtrate is completely free of phosphotungstic acid. The precipitated phosphotungstate was removed by centrifugation and by filtration of the supernatant solution through a glass filter of fine porosity, and the filtrate was concentrated *in vacuo* to a small volume (about 2 ml. per gm. of protein). To the concentrate methanol (0.5 ml. per gm. of protein) and 18 N H_2SO_4 (1.5 ml. per gm. of protein) are added (in this order, or the

methanol and the sulfuric acid are mixed first, and the mixture is added to the concentrate). Any precipitate appearing at this stage is removed by filtration, washed with water, and discarded. The washings and the sulfuric acid digest are then concentrated to the original volume *in vacuo*.[1] The sulfuric acid mixture was then refluxed for 0.5 hour. Longer digestion does not alter the final yields, nor does it affect the procedure in any way. The brown digest was diluted with water (to 20 ml. per gm. of protein started) and filtered. The filtrate was warmed on a steam bath, and to it a hot solution of phosphotungstic acid was added (1 gm. in 1.2 ml. of water per gm. of the protein used). The mixture was placed in a refrigerator for about 2 hours, and the precipitate was removed by filtration through a glass filter of medium porosity and washed with water until the filtrate was free of sulfate ($BaCl_2$ test). The washed precipitate was then thoroughly washed with five portions of about 100 ml. each of 95 per cent ethanol,[2] then with ethyl ether, dried, and weighed. To the weighed phosphotungstate 90 per cent aqueous acetone was added (about 0.5 ml. per gm. of protein started), and the mixture was thoroughly triturated, then filtered. The undissolved residue was again triturated with two additional similar portions of aqueous acetone, and the acetone extracts were combined. The undissolved residue was washed with ethyl ether, dried, and weighed. The difference in the weight of the phosphotungstate before and after trituration with acetone gives the weight of the methionine methylsulfonium phosphotungstate that dissolved in the acetone. For every gm. of the phosphotungstate dissolved in the aqueous acetone 1 ml. of 1 M tetraethylammonium bromide was added to the combined acetone extracts, followed by 4.5 ml. of water per gm. of protein used. The mixture, after stirring, was centrifuged. The supernatant solution was filtered through a glass filter of fine porosity, and the pH of the filtrate was adjusted to 5 with 1 M ammonium hydroxide. The solution was then decolorized with carboraffin in the cold, filtered through a glass filter of fine porosity, and evaporated to near dryness *in vacuo* at 25–30°. A 10 ml. portion of methanol was added to the concentrate; the methanol was distilled *in vacuo*, and the procedure of addition and distillation of methanol was repeated twice more. The yellowish oil was transferred quantitatively to a centrifuge tube with minimal amounts of methanol (about 0.5 ml. per gm. of protein started), and the distillation flask was rinsed out with absolute ethanol (2 ml. per gm. of protein started). The ethanolic rinse was added to the centrifuge tube containing the methanol solution of the sul-

[1] By the "original volume" is meant 2 ml. per gm. of the protein used.

[2] Ethanol removes a large proportion of the phosphotungstates, including those formed from cystine, homocystine, and cystathionine, if the latter are present in or added to the hydrolysates. Thorough washing with ethanol is essential at this step of the procedure.

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fonium salt. The alcoholic solution of the salt was allowed to crystallize in a refrigerator for 2 to 3 hours, and the precipitated material was separated by centrifugation, washed with absolute ethanol, then recrystallized either from hot methanol or from methanol by the addition of ethanol. The final product was washed with absolute ethanol, followed by ethyl ether, and dried *in vacuo* at room temperature. The isolated L-methionine methylsulfonium bromide is somewhat hygroscopic, and prolonged exposure to moist air should be avoided. In moist air it forms a glassy transparent mass without apparent decomposition. The dry salt can be recovered by recrystallization from methanol. The yields of methionine methylsulfonium bromide varied, in several hundred isolations performed to date, from 14 to 17 mg. per gm. of protein used, m.p. 136–138° (open capillary; sintering due to decomposition to dimethyl sulfide (*cf.* (2)). The isolated material gave satisfactory values for methionine methylsulfonium bromide on elementary analysis. Methionine methylsulfonium bromide was isolated from tissues after administration to animals of nine different precursors of methionine labeled with C^{14} or S^{35} and chromatographed on Whatman paper No. 1 with three different solvent systems (*t*-butanol-formic acid-water (7:1.5:1.5), *n*-butanol-acetic acid-water (4:1:1), and *n*-butanol-95 per cent ethanol-2 N HCl (4:1:2)). Single spots were obtained in all cases, and the R_F values agreed well with those reported (5). Pyridine, phenol, and lutidine were found to be unsatisfactory, owing to the decomposition of methionine methylsulfonium in these alkaline reagents. Thus, within the limits of accuracy of paper chromatographic technique, the isolated methionine methylsulfonium bromide appears to be reasonably free of extraneous material.

To test the possibility of an exchange of the methyl group of methionine and of methionine methylsulfonium with methyl of methanol when the latter is present in excess in the sulfuric acid digestion mixture, the following experiment was performed. Methionine methylsulfonium-CH_3-C^{14} was prepared from methionine-CH_3-C^{14} by digestion of the latter in 18 N H_2SO_4 in the presence of non-isotopic methanol by the adaptation of the procedure of Lavine *et al.* (2). 1 mmole of radiomethionine and 1 mmole of radiomethionine methylsulfonium bromide were digested separately in 18 N H_2SO_4 in the presence of 1, 2, 4, or 8 mmoles of non-isotopic methanol, and radiomethionine methylsulfonium bromide was isolated in each case in the usual way. The products were then assayed for radioactivity. From the results in Table I it can be seen that no apparent exchange of the methyl group of either methionine or of methionine methylsulfonium with that of methanol took place under the conditions employed.

The possible interference by tissue cystine with the purity of the methionine methylsulfonium salt which is isolated from the animal tissues and the

completeness of the removal of the product which is formed from cystine on digestion of the latter in strong sulfuric acid in the presence of methanol were tested as follows: To 10 gm. of rat "protein" hydrolysate in 6 N HCl 0.2 mmole of cystine-S^{35} was added (activity of cystine-S^{35}, 2.5×10^6 c.p.m. per mmole). Methionine methylsulfonium bromide was then isolated from the digest as described. Negligible activity in the sulfonium salt was obtained (7 ± 4 c.p.m. per mmole). A maximal contamination by cystine is, therefore, about 0.014 per cent under the conditions of isolation of methionine methylsulfonium salt from animal tissues. A single spot of R_F 0.35 was obtained on Whatman paper No. 1 with *n*-butanol-95

TABLE I

*Effect of Excess Methanol on Activity of Methyl Groups of Methionine-CH_3-C^{14} or of Methionine Methylsulfonium-CH_3-C^{14} on Digestion in 18 N H_2SO_4**

	Total activity	Total activity of methionine methylsulfonium bromide			
		Equivalents of methanol used in digestion			
		1	2	4	8
	c.p.m.	c.p.m.	c.p.m.	c.p.m.	c.p.m.
Methionine-CH_3-C^{14}	14,500	14,560	14,455	14,600	14,530
Methionine methylsulfonium-CH_3-C^{14}	6,000	5,970	5,800	5,930	5,950

* 1 mmole of the radiocompound was digested in 18 N H_2SO_4 in the presence of 1, 2, 4, or 8 mmoles of methanol for 0.5 to 1 hour, and methionine methylsulfonium bromide was isolated in each case as described in the text.

per cent ethanol-2 N HCl (4:1:1) on the methionine methylsulfonium bromide which was isolated from the digests containing cystine-S^{35}.

Methionine methylsulfonium bromide, added to animal protein digests, could be recovered in the first precipitation of the digests with phosphotungstic acid. The possible presence of preformed sulfonium salts in animal tissues could thus be detected by analysis of the phosphotungstates, which are obtained in the first step of the procedure, for the sulfonium salts. The possibility of formation *in vivo* of methionine methylsulfonium from methionine was tested as follows: Rats received methionine-CH_3-C^{14} and were sacrificed 5 to 20 hours later, and the proteins were digested in HCl as described. The first phosphotungstate precipitate, which was obtained on adding phosphotungstic acid to the deacidified and clarified digest, was examined for radioactivity. None was present. Thus, methionine-CH_3-C^{14}, present in the digest, is not precipitable with phosphotungstic acid, and no detectable amount of methionine methylsulfonium-CH_3-C^{14} was present in the digest. When methionine methylsulfonium-CH_3-C^{14}

was digested together with rat proteins in 6 N HCl for 6 hours and the deacidified and clarified digest was treated with phosphotungstic acid, the phosphotungstate was radioactive, and pure methionine methylsulfonium-CH_3-C^{14} was recovered from the phosphotungstate. The apparent absence of methionine methylsulfonium in rat proteins, following the digestion of the proteins with HCl, does not, however, eliminate the possibility of formation of methionine methylsulfonium *in vivo*, particularly because this sulfonium salt has been isolated from several plant sources (5, 6), and because it acts *in vivo* as a potent source of its methyl group in animals (7) and in molds (8).

SUMMARY

The procedure of Floyd-Lavine has been adapted to the isolation of methionine as methionine methylsulfonium bromide from animal proteins, and the procedure is suitable in tracer studies involving the metabolism of methionine.

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INHIBITORY ACTION OF *meso*-TARTRATE ON PYRUVATE OXIDATION

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Naturally occurring tartrates are apparently not metabolized by mammalian tissues (1–5), the disappearance of ingested (though not injected) tartrates being ascribed to their metabolism by intestinal microorganisms (4, 5). The tartrates, however, have a little biological activity, D-tartaric acid being weakly ketogenic in the presence of rat liver slices (6) and depressing their respiration. *meso*-Tartrate (0.07 M) is highly inhibitory to *Escherichia coli* lactic dehydrogenase, less inhibitory to *E. coli* succinic dehydrogenase, and is a more effective inhibitor of both enzymes than D-tartrate (7). The stereoisomers of tartaric acid may be differentiated by their effects on prostate phosphatase (8, 9), which is stimulated by the D isomer, strongly inhibited by the L isomer, and feebly inhibited by the meso isomer.

The present report describes a specific inhibitory action of *meso*-tartaric acid on pyruvate metabolism in rat kidney slices and in rat kidney mitochondria.

Methods and Materials

A local strain of inbred hooded rats was used throughout. For experiment, the animals were stunned and bled and the excised tissues placed in cracked ice. Slices of rat kidney cortex were cut with a Stadie-Riggs tissue slicer (Arthur H. Thomas Company) and immediately placed on a Petri dish containing cracked ice. Prior to use, the slices were blotted, weighed on a torsion balance, and placed directly in chilled Warburg manometric flasks. Standard manometric techniques were adopted.

The incubation medium was composed as follows: 0.13 M NaCl, 0.005 M KCl, 0.0013 M KH_2PO_4, 0.0013 M $MgSO_4$, 0.01 M sodium phosphate buffer, pH 7.4. Allowance was made for the addition of the sodium salts of organic acids by addition of a correspondingly lower concentration of sodium chloride.

Mitochondria were prepared by the method of Schneider (10) with 0.25 M sucrose and were incubated in the medium described by Judah and Rees (11).

The Q_{O_2} values were calculated from the oxygen uptake at 37° measured over the first 60 minute period after thermal equilibration, assuming 80 per cent moisture in the slice.

Results

Experiments with Rat Kidney Cortex Slices—Sodium *meso*-tartrate inhibits the respiration of rat kidney cortex slices in the presence of sodium pyruvate (Table I), but the D and DL isomers are without effect. None of the three tartrates affects the respiration in the presence of DL-malate,

TABLE I

Effect of D-, DL-, *and meso-Tartrate on Respiration of Rat Kidney Cortex Slices in Presence of Various Substrates*

Substrate	$-Q_{O_2}$* in presence of			
	No inhibitor	0.02 M sodium D-tartrate†	0.02 M sodium DL-tartrate	0.02 M sodium *meso*-tartrate
None	18.4	17.3	15.4	12.1
0.02 M sodium pyruvate	27.2	26.0	25.1	14.7
0.02 " " citrate	27.2	27.7	25.5	25.8
0.02 " " α-ketoglutarate	26.7	26.7	25.1	24.9
0.02 " " DL-malate	28.4	27.8	24.7	24.6

* The $-Q_{O_2}$ values quoted are the averages obtained from at least three separate determinations obtained over 5 to 65 minutes.

† All values of concentrations refer to the final concentration in the medium.

TABLE II

Effect of Various Concentrations of meso-Tartrate on Respiration of Rat Kidney Cortex Slices in Presence of Pyruvate

Sodium pyruvate	$-Q_{O_2}$ in presence of *meso*-tartrate			
	0	0.002 M	0.007 M	0.02 M
M				
0	16.5	13.2	11.6	11.6
0.02	25.5	22.4	19.5	15.4

citrate, or α-ketoglutarate. Similarly, tartronic (hydroxymalonic) acid has no effect on the respiration of rat kidney cortex slices in the presence or absence of pyruvate. Although a marked inhibitory effect of sodium *meso*-tartrate on respiration obtains at a concentration of 0.02 M, some effect is obvious even at one-tenth this concentration, particularly in the absence of added substrate (Table II).

Pyruvate disappearance in the presence of rat kidney cortex slices is markedly inhibited by the presence of *meso*-tartrate. In a typical experiment, incubation of rat kidney cortex slices with 0.02 M sodium pyruvate

at 37° resulted in the disappearance in 1 hour of 16.4 μmoles of sodium pyruvate per 100 mg. wet weight of tissue. In the presence of 0.02 M sodium *meso*-tartrate, the disappearance of sodium pyruvate in 1 hour was only 6.95 μmoles per 100 mg. wet weight of rat kidney cortex, an inhibition of 60 per cent thus being effected.

The addition of 2×10^{-2} M sodium fumarate completely reverses the inhibition of respiration of rat kidney cortex slices in the presence of pyruvate produced by *meso*-tartrate, and 7×10^{-4} M sodium fumarate partially reverses the inhibition. Typical results are shown in Table III. DL-Malate is less effective than fumarate in this respect, but it is more effective than citrate at equivalent concentrations (Table III).

TABLE III

Reversal by Fumarate, Citrate, and Malate of meso-Tartrate Inhibition of Pyruvate Oxidation in Rat Kidney Cortex Slices

Further additions	$-Q_{O_2}$			
	No substrate	0.02 M sodium pyruvate	0.02 M sodium *meso*-tartrate	0.02 M sodium *meso*-tartrate + 0.02 M sodium pyruvate
None	16.7	29.0	11.0	14.2
0.0007 M sodium fumarate	20.1	26.7	14.0	19.5
0.002 " " "	20.3	25.8	15.3	25.4
0.002 " " citrate	21.4	28.6	15.3	20.7
0.002 " " DL-malate	22.1	27.4	15.1	23.2

Experiments with Rat Kidney Mitochondria—The rate of oxidation of α-ketoglutarate, fumarate, or DL-malate by rat kidney mitochondria is inhibited to a small extent by all three tartrates. Typical results with α-ketoglutarate as substrate are shown in Table IV. *meso*-Tartrate inhibits the rate of oxygen uptake and its effect increases with time until, after 1 hour, 48 per cent inhibition is obtained. DL-Tartrate has a similar effect, but after 1 hour it inhibits the rate of oxygen uptake by only 16 per cent. Addition of D-tartrate produces a constant inhibition of approximately 25 per cent. Since similar small inhibitory effects occur with fumarate and DL-malate as substrates, it follows that the tartrates have but little direct effect on the oxidations involved in the tricarboxylic acid cycle.

The agent used for the initiation of mitochondrial oxidations influences the effect of *meso*-tartrate on the oxidation of pyruvate. The rate of oxidation of pyruvate in the presence of 0.0007 M DL-malate is affected by the tartrates in a manner similar to their effects on the rates of oxidation of

α-ketoglutarate, fumarate, or DL-malate. Thus, the oxidation of pyruvate by pyruvic oxidase is but little affected in the mitochondrial system by the

TABLE IV

Effect of D-, DL-, *and meso-Tartrate on Oxidation of α-Ketoglutarate by Rat Liver Mitochondria*

Each vessel contained the basic medium described by Judah and Rees (11) together with 0.7 ml. of suspension of mitochondria in 0.25 M sucrose and 20 μmoles of potassium α-ketoglutarate in 3 ml. Gas phase, air; temperature, 37°.

Further additions	O_2 taken up, μl.		
	0–20 min.	20–40 min.	40–60 min.
None	165	149	139
20 μmoles potassium *meso*-tartrate	146 (12)*	102 (32)	72 (48)
20 " " D-tartrate	123 (25)	116 (22)	107 (23)
20 " " DL-tartrate	165 (0)	139 (7)	117 (16)

* The figures in parentheses refer to the percentage inhibition of oxygen uptake.

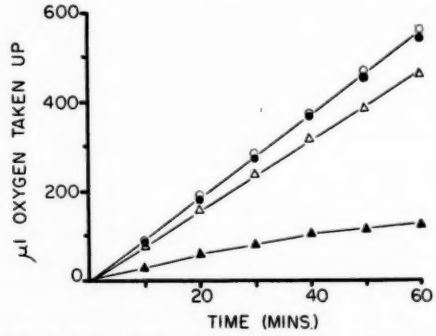

FIG. 1. The effect of *meso*-tartrate on the oxidation of pyruvate by rat kidney mitochondria. All vessels contained the standard incubation medium (see "Methods and materials") with the following additions (in micromoles) (final volume 3 ml.): ○, pyruvate 20, DL-malate 2; ●, pyruvate 20, bicarbonate 9; △, pyruvate 20, DL-malate 2, *meso*-tartrate 20; ▲, pyruvate 20, bicarbonate 9, *meso*-tartrate 20. In all control vessels, without pyruvate or "primer," less than 50 μl. of oxygen was taken up in the experimental period.

tartrates, and this observation is confirmed by the fact that the oxidation of pyruvate by brain homogenates, as shown by respiratory activities, is also unaffected by the tartrates.

It is already known (12) that both bicarbonate and malate stimulate the oxidation of pyruvate by rabbit kidney "cyclophorase" preparations. Preliminary experiments indicated that, with preparations of crude rat

liver mitochondria, malate is not required to initiate pyruvate oxidation. With twice washed rat kidney mitochondria, however, a "primer" is necessary to initiate pyruvate oxidation, but bicarbonate is unable to act in this capacity. When the number of washings of the rat kidney mitochondria in isotonic sucrose solution is reduced to one, bicarbonate is as effective as malate in initiating the oxidation of pyruvate. Under these conditions, it is found that sodium *meso*-tartrate has little effect on the malate-stimulated oxidation of pyruvate, but it produces a marked inhibition of the bicarbonate-stimulated oxidation of pyruvate by rat kidney mitochondria. Typical results are shown in Fig. 1.

DISCUSSION

As the oxidation of pyruvate in rat brain homogenate, or in rat kidney or liver mitochondria in the presence of DL-malate, is unaffected by the presence of sodium *meso*-tartrate, it is clear that pyruvic oxidase activity in these tissues is not inhibited by *meso*-tartrate. The respiration of rat kidney cortex slices in the presence of pyruvate and the metabolism of pyruvate in this tissue are, however, markedly inhibited by *meso*-tartrate. Therefore, the site of action of this substance must lie with the further oxidation of the acetyl moiety (acetyl coenzyme A) derived from the pyruvate. *meso*-Tartrate, on the other hand, has no inhibitory effect on the oxidation by rat kidney cortex slices of members of the tricarboxylic acid cycle, for no diminution of respiration of this tissue in the presence of citrate, α-ketoglutarate, or DL-malate is observed. It follows that the inhibition must be concerned with the condensation reaction leading to citrate synthesis.

The complete reversal of the inhibitory effect of *meso*-tartrate on pyruvate oxidation by 0.002 M fumarate and the partial reversal by DL-malate and citrate make it likely that *meso*-tartrate acts by inhibiting the formation of dicarboxylic acid from pyruvic acid in rat kidney cortex slices.

Confirmation of this suggestion was obtained with mitochondrial preparations. In once washed rat kidney mitochondria, the incorporation of bicarbonate leads to rapid oxidation of pyruvate. This oxidation was found to be extremely sensitive to the presence of *meso*-tartrate, in contrast to the malate-stimulated oxidation of pyruvate. A further washing of the rat kidney mitochondria with isotonic sucrose markedly decreases the ability of bicarbonate to stimulate the respiratory activity of this system.

This evidence is consistent with the conclusion that *meso*-tartaric acid is a specific inhibitor of the incorporation of CO_2 into the dicarboxylic acids of rat kidney. Such an inhibition would lead to the suppression of pyruvate oxidation in rat kidney cortex slices or mitochondria by preventing the formation of the oxalacetate required for oxidation, by the citric acid cycle, of the acetyl coenzyme A derived from pyruvate.

SUMMARY

1. *meso*-Tartrate is a specific inhibitor of pyruvate oxidation in slices of rat kidney cortex. The inhibition of oxygen uptake in the presence of added pyruvate brought about by *meso*-tartrate is completely reversed by addition of trace amounts of fumarate and partially reversed by small amounts of DL-malate or citrate.

2. No oxidation of pyruvate takes place in rat kidney mitochondria unless a "primer" is added. When malate is used as a "primer," little inhibition by *meso*-tartrate of pyruvate oxidation occurs.

3. Bicarbonate can serve as a "primer" of pyruvate oxidation in once washed preparations of rat kidney mitochondria. In this system, pyruvate oxidation is inhibited by the presence of *meso*-tartrate.

4. It is suggested that *meso*-tartrate inhibits the incorporation of carbon dioxide into pyruvate in rat kidney and thus, by suppressing dicarboxylic acid formation, prevents oxidation of pyruvate.

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STUDIES ON THE CHEMISTRY OF THE CORD FACTOR OF MYCOBACTERIUM TUBERCULOSIS

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A lipide component of the tubercle bacillus, which was termed "cord factor" (1–4), has been described in previous publications. This bacterial constituent is characterized by its exclusive occurrence in strains of *Mycobacterium tuberculosis* which have the ability to multiply *in vivo*, by a particular delayed toxicity for mice upon repeated injections of small amounts (2.5 to 5 γ of pure preparations), and by a definite rôle it plays in the virulence of the tubercle bacillus (5).

The present paper describes methods for obtaining highly purified preparations of cord factor, and it shows that cord factor is present in different virulent strains of *M. tuberculosis* of both the human and the bovine type. It presents a partial structural analysis of cord factor, showing that the compound is an ester of mycolic acid containing 1 molecule of glucose, the latter being attached through a glucoside linkage to an as yet unidentified aliphatic fragment. A detailed description of the biological properties of the cord factor will be reported elsewhere (6).

Anderson's pioneer work on mycobacterial lipides has made use of solvent fractionation methods (7), and fractionation by chromatography has been profitably introduced in this field by Asselineau and Lederer (8). The present workers have benefited from both these methods and, in addition, made extensive use of infra-red spectroscopy[1] as an aid in the identification of microquantities of lipides. The combined application of the three methods proved to be an excellent tool in the isolation and structural analysis of high molecular lipides of this class.

EXPERIMENTAL

Isolation and Purification of Cord Factor from Various Strains of Virulent Tubercle Bacilli

Bacteria—The bacilli were grown as surface cultures on a modified Lockemann medium (9) and harvested after about 3 weeks as described

[1] The authors are greatly indebted to Dr. R. C. Gore, and Mr. N. B. Colthup, of the American Cyanamid Company, Stamford, Connecticut, for the courtesy of running a great number of infra-red spectra and for their invaluable help in interpreting the results.

previously (3). The following strains were used for the preparation of cord factor: (*a*) H37-Rv and (*b*) Brévannes, both virulent human strains; (*c*) PN, DT, C, a mixture of virulent human strains used in the production of tuberculin;[2] (*d*) Vallée, a virulent bovine strain; and (*e*) a variant of H37-Rv, resistant to isonicotinic acid hydrazide, designated "Ciba."[3]

Extraction and Isolation of Purified Wax—All bacilli were extracted in the viable state, with the exception of bacilli (*b*) and (*c*), which were obtained as heat-killed cultures. Strain Brévannes was selected for the production of larger quantities of cord factor in most of the present studies.[4] For practical reasons, steam-killed, 3 week-old cultures were used for all large scale extractions of the strain Brévannes.

An adaptation of Anderson's extraction scheme, as previously described (3), was employed with some minor modifications. In Anderson's procedure (10), the bacilli are defatted by preliminary extraction with ether-ethanol 1:1 (volume by volume) and subsequently extracted with chloroform. It was found that the cord factor-containing fraction, Wax C (11), of the chloroform extract was soluble in ether-methanol 1:1, but insoluble in a mixture of 1 part ether and 2 parts methanol. In order to prevent losses of cord factor-containing material, we used a mixture of 1 part ether and 2 parts methanol for the preliminary extraction.

Before the chloroform extraction, the bacilli were washed with acetone, dried, and pulverized. The purified wax obtained according to Anderson is usually contaminated with yellow pigments. These pigments were effectively removed by washing the purified wax with cold acetone on the filter.

The purified wax was separated into fractions of Waxes C and D by fractional precipitation with ether-methanol, as previously described (3). It is of the utmost importance to obtain a clean separation of Wax C from Wax D. Wax C (m.p. 40–50°) consists of low melting esters, including cord factor, while Wax D (m.p. 160–240°) is composed of high melting lipopolysaccharides (11). The presence of lipopolysaccharides in Wax C not only interferes with its chromatographic purification but also gives rise to misleading results in the chemical analyses of its components.

Chromatographic Purification of Cord Factor—For the chromatographic procedures, the following adsorbents were used: magnesium silicate-Celite

[2] The authors wish to thank Dr. H. D. Piersma of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for the generous supply of these bacterial cultures.

[3] Obtained through the courtesy of Dr. R. L. Mayer, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

[4] We are indebted to Dr. R. L. Mayer, Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for providing us with large amounts of bacterial cultures.

545 (12)[5] and silica gel "Davison." The separation of the components was followed by melting point and acidity determinations as well as infrared spectroscopy. The particulars of the techniques have been described (3).

Separation of Cord Factor and Mycolic Acid on Silica Gel—Pure cord factor is difficult to obtain from Wax C of old bacterial cultures because of

TABLE I

Chromatogram I. Chromatography of Ether-Methanol Fractions Obtained after Chromatography of Wax C from Strains PN, DT, C on Magnesium Silicate-Celite

310 mg. of material were adsorbed on 30 gm. of silica gel.

Fraction No.	Eluted with 30 ml. of		M.p.	C.mm. 0.1 N MeONa per mg.	$[\alpha]_D^{25}$
		mg.	°C.		*degrees*
1	Petroleum ether	30.4			
2	" "	17.2			
3	" "	15.2	36–37	0.5	+31
4	" "	10.8			
5	" "	0.6			
6–11	" ether-benzene 1:1	6.1			
12	Benzene-ether 1:1	116.4			
13	" 1:1	32.5			
14	" 1:1	14.2			
15	" 1:1	7.7	52–54	5.9	+9
16	" 1:1	7.9			
17	" 1:1	6.3			
18	Ether containing 5% MeOH	5.0			
19	" " 5% "	6.8			
20	" " 5% "	6.1	44–46	2.8	+26
21	" " 5% "	2.2			
22	" " 10% "	13.0			
Recovered		298.4 = 96%			

the latter's high content of free mycolic acid which is difficult to separate from cord factor. On magnesium silicate-Celite, repeated chromatographies are needed and the separation is still incomplete (3). Subsequent chromatography on silica gel permitted a more effective separation of the two components, as seen from a typical example in Table I. The toxic component, in contrast to its strong adsorption on magnesium silicate, is only slightly adsorbed on silica gel and eluted in the very beginning with petroleum ether.[6]

[5] We are indebted to Johns-Manville, New York, for a gift of Celite 545.

[6] The mycolic acid fraction eluted with benzene-ether seems to be contaminated with toxic material, since its acidity (5.9 c.mm. of 0.1 N MeONa per mg.) is lower

The toxic fraction eluted from the silica gel column with petroleum ether still contained traces of acidity (Table I) which could not be completely removed by repeated chromatographies. Esterification with diazomethane yielded a completely neutral material which, after chromatography, was slightly acid again, suggesting that small amounts of mycolic acid were liberated on the column.

The sample of cord factor obtained by chromatography on silica gel had the physicochemical properties listed in Table III.

Infra-Red Spectrum (*Fig. 1, Curve 1*)—The spectrum shows a series of well defined bands all associated with the toxic fractions (3). The C—O

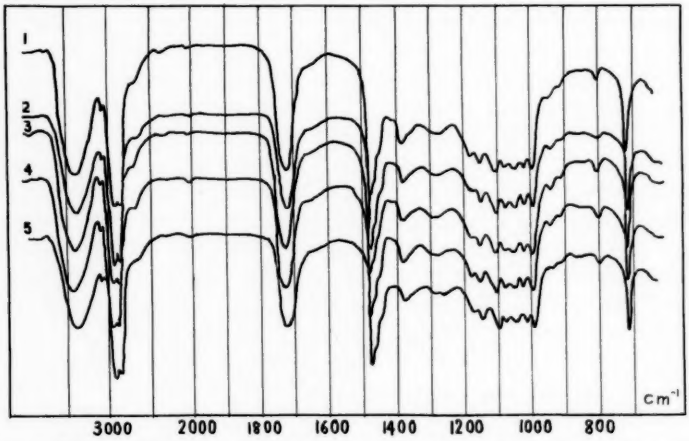

FIG. 1. Infra-red spectra of cord factor preparations (taken from melts) from five different bacterial strains. Curve 1, PN, DT, C; Curve 2, H37-Rv; Curve 3, Brévannes; Curve 4, Vallée; Curve 5, "Ciba."

stretching region, 1200 to 800 cm.$^{-1}$, which is commonly used for the identification of closely related compounds, is highly characteristic for purified cord factor samples. The exact positions of the absorption maxima are 3350, 2900, 2840, 1715, 1470, 1380, 1275, 1170, 1150, 1100, 1075, 1050, 1020, 995, 915, 805, and 720 cm.$^{-1}$. Upon repeated injections in mice, this sample displayed characteristic toxicity (5). The data mentioned above were found to be reproducible and consistent for various preparations obtained in different ways in this laboratory, as well as in the laboratory of Dr. E. Lederer in Paris.

than the theoretical value of 7.7 c.mm. of 0.1 N MeONa per mg. and its optical rotation ($[\alpha]_D$ +9°) is higher than the value reported for mycolic acid ($[\alpha]_D$ +1.8°) (7). For unknown reasons, a small amount of toxic material was always eluted in the tail fractions with ether methanol.

Isolation of Cord Factor from Different Strains—As has been pointed out previously (3), old cultures of tubercle bacilli are a poor source of cord factor. Thus, Wax C from the 12 week-old bacilli (*c*) yielded only 1 to 3 per cent, while 10 per cent or more was obtained from 2 to 3 week-old bacilli. The separation of cord factor from Wax C of the H37-Rv strain

TABLE II

Chromatogram II. Chromatography of 790 Mg. of Wax C from Strain H37-Rv Dissolved in Petroleum Ether Adsorbed on 60 Gm. of Magnesium Silicate-Celite

Fraction No.	Eluted with 50 ml. of		M.p.	C.mm. of 0.1 N MeONa per mg.
		mg.	°C.	
1	Petroleum ether	237.8	29–31	
2	" "	142.6		
3	" "	46.7		0
4	" "	21.0		
5	" "	25.5	32–33	
6	" ether-benzene 1:1	84.2	40–45	0
7	" " 1:1	20.8	48–55	0
8	" " 1:1	8.0	35–45	
9	" " 1:1	6.5		
10	" " 1:1	5.8		
11	" " 1:1	5.2		
12	Benzene-ether 1:1	9.8	54–60	
13	" 1:1	6.4		5.0
14–16	" 1:1	4.1		
17*	Ether containing 10% MeOH	67.7	41–42	1.0
18	" " 10% "	25.4		
19	" " 10% "	6.7		
20	" " 10% "	4.6		
21–32	" " 10% "	12.0		
Recovered		740.8 = 94%		

* The fractions representing cord factor are given in bold-faced type.

is shown in Chromatogram II (Table II). The ether-methanol eluate yielded cord factor of high purity after only one chromatography on magnesium silicate-Celite, since the Wax C of these bacilli contained little or no free mycolic acid. Similar results were obtained in the chromatography of Wax C from Brévannes, Vallée, and "Ciba" strains. Fig. 1 shows that the infra-red spectra of the toxic components obtained from five different bacterial strains are identical. Other data pertaining to these fractions are summarized in Table III.[7]

[7] Analyses by the Schwarzkopf Microanalytical Laboratory, Middle Village, New York.

Thus the same toxic fraction could be isolated from a variety of virulent strains of tubercle bacilli. The yields range from 5 to 20 per cent of Wax C. This is of particular significance in view of the biological rôle played by cord factor in tuberculous infection (6). In contrast, the nature of the non-toxic components of Wax C varies markedly with the different sources.[8]

Chemical Degradation of Cord Factor

Alkaline Hydrolysis of Cord Factor—190 mg. of cord factor (Brévannes) were suspended in a solution of 5 cc. of isopropanol, 0.1 cc. of water, and 100 mg. of KOH. The solution was refluxed for 1 hour under a stream of nitrogen which was passed through a trap containing 5 cc. of a 0.1 N HCl solution. After about 10 minutes of heating, a clear solution of yellowish

TABLE III

Properties of Cord Factor Preparations Isolated from Various Strains of Virulent Tubercle Bacilli

Strain	M.p.	$[\alpha]_D^{25}$	Infra-red spectrum No.	C	H	N	N—CH_3
	°C.	degrees		per cent	per cent	per cent	per cent
PN, DT, C	36–37	+31	1	76.3	12.5	0.8	1.0
H37-Rv	41–42	+30	2	76.4	12.7		1.0
Brévannes	40–41	+31	3	76.9	12.3	0.7	1.4
Vallée	38–39	+29	4				
"Ciba"	42		5				

color was obtained. At the end of the heating period, the solution was cooled and neutralized with 2 cc. of 1 N HCl.

Test for Volatile Bases—Microtitration of an aliquot of the 0.1 N HCl trap solution showed no decrease in acidity. Lyophilization of the trap solution left only an insignificant trace of a hygroscopic residue.

Isolation of Mycolic Acid As Lipide Moiety—After addition of 5 cc. of water, the acidified solution obtained after alkaline hydrolysis was repeatedly extracted with an excess of petroleum ether. The petroleum ether extracts were taken to dryness, and the lipide residue was dissolved in a small volume of ether and precipitated with methanol. The precipitate was filtered, washed with methanol, and dried *in vacuo* at room temperature; 158 mg. of a white powder, m.p. 54–55°, were obtained. The neutral equivalent was 1275 ± 25. Found, C 81.49, H 13.30, OCH_3 1.71.[9]

[8] Noll, H., paper to be published.

[9] The theoretical value corresponding to one methoxyl group per molecule is 2.4 per cent. As pointed out by Dr. Lederer (personal communications), the methoxyl determinations on mycolic acids are often erroneous. On the other hand Aebi *et al.* (13) have shown that preparations of α-mycolic acid obtained from strain Brévannes

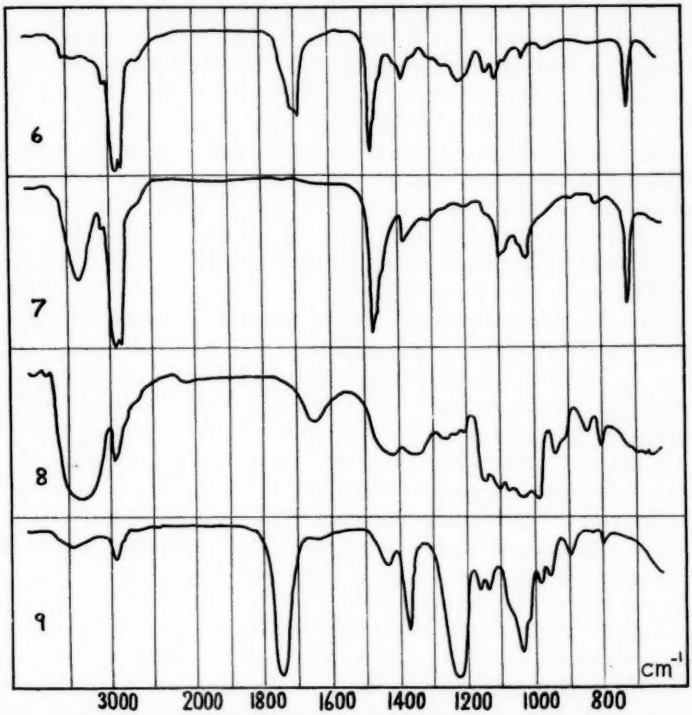

FIG. 2. Infra-red spectra of degradation products of cord factor. Curve 6, α-mycolic acid; Curve 7, α-mycolic alcohol; Curve 8, non-reducing carbohydrate fragment; Curve 9, acetylated non-reducing carbohydrate fragment. Spectra in Curves 6 and 7 were taken from melts; those in Curves 8 and 9 from smears.

These values agree with those published by Aebi *et al.* for α-mycolic acid Brévannes (13). The infra-red spectrum (Fig. 2, Curve 6) is identical with that of a sample of α-mycolic acid Brévannes obtained from Dr. Lederer.

consist of a mixture of two acids which cannot be separated by chromatography. The fact that one of these acids contains one methoxyl group while the other has a hydroxyl in place of the methoxyl group explains the low methoxyl values obtained by analysis of the mixture. It seems conceivable, therefore, that both of these acids can take part in the synthesis of cord factor. Preparations of cord factor obtained from the strains PN, DT, C yielded α-mycolic acid, which was devoid of methoxyl. In their extensive studies on the chemistry of mycolic acid (25), Dr. Lederer and co-workers have shown that the mycolic acids elaborated by different strains of *Mycobacteria* vary to some extent. Dr. Asselineau and Dr. Lederer (personal communications) also found that cord factor preparations obtained from various strains of virulent tubercle bacilli yielded different mycolic acids upon alkaline hydrolysis.

Isolation of Water-Soluble Moiety—The acidified water solution obtained after alkaline hydrolysis was deionized by passing through a mixed anion-cation exchange resin (Amberlite MB-3) and concentrated *in vacuo* to a volume of 2 cc. This concentrated solution was divided into two portions of 1 cc. each, designated in the following as Solutions 1 and 2. Solution 1 was lyophilized, yielding a white fluffy residue and turning into a sirup at atmospheric pressure. About 10 mg. of this hygroscopic residue were recovered and 1 mg. dissolved in 100 c.mm. of water which formed a cloudy solution. Aliquots of 10 c.mm. each were withdrawn for the following tests: (1) the reduction test according to Nelson (14), in which no reducing action was detected; (2) the sulfuric acid test according to Mendel *et al.* (15), in which the solution obtained after heating with concentrated sulfuric acid had a pink color. The spectrum was measured with a Beckman model DU spectrophotometer in the ultraviolet region between 215 and 350 mμ and in the visible region between 400 and 600 mμ. A sample of 100 γ of glucose per cc. served as a control. The spectra of the alkaline hydrolysate and of the glucose solution were identical, the characteristic maxima being shown at 255, 312, and 520 mμ. The extinction values of the alkaline hydrolysate were equal to those of 100 γ of glucose. According to Mendel *et al.*, the pink color with a maximum at 520 mμ is specific for glucose, fructose, and their polymers.

The fact that the water-soluble moiety resulting from alkaline hydrolysis of cord factor gives no reducing action, but a positive glucose test with concentrated sulfuric acid, suggests that glucose is liberated under the strongly hydrolytic conditions of the test. It was indeed found that subsequent acid hydrolysis results in the liberation of a reducing sugar which was identified as glucose, as described in the following section.

Ultraviolet Spectrum of Water-Soluble Moiety—Except for the unspecific end-absorption at 215 mμ, no absorptions were detected between 220 and 400 mμ.

Infra-Red Spectrum—The infra-red spectrum of the hygroscopic residue obtained after lyophilization is pictured in Fig. 2, Curve 8. Taken from a smear, this spectrum shows the typical absorptions of sugars; *i.e.*, a strong bonded —OH band at 3350, broad O—H bending absorptions between 1500 and 1300, and a system of well defined bands in the C—O stretching regions with maxima at 1150, 1105, 1080, 1035, 990, and 940 cm.$^{-1}$. The broad absorption between 1650 and 1600 cm.$^{-1}$ is probably due to water. The CH_2 band at 2940 cm.$^{-1}$ seems somewhat deeper than usual in sugars, indicating the presence of an aliphatic residue. Although the spectrum is typical for a carbohydrate, it is not identical with any of the spectra of the sugar and sugar derivatives published by Kuhn (16). This is not surprising, since the carbohydrate moiety obtained after alkaline hydrolysis of cord factor is a non-reducing derivative of glucose, and

the reducing sugar is liberated only after subsequent acid hydrolysis as described below.

Acid Hydrolysis of Non-Reducing Carbohydrate Fragment Obtained after Alkaline Hydrolysis of Cord Factor—To 1.0 cc. of Solution 2, 1.0 cc. of 3 N HCl was added, and the resulting solution heated in a sealed tube for 4 hours in a boiling water bath. An aliquot of 0.05 cc. of the hydrolyzed solution was withdrawn for a reduction test according to Nelson (14), which was strongly positive. Colorimetric determination gave a value corresponding to a total of 8.8 mg. of glucose in Solution 2. The acidic solution was divided into two equal portions, Solutions 3 and 4. Solution 3 was made alkaline with NaOH and extracted with ether. The ether extract was treated with dry HCl and a cloudy precipitate obtained which, after evaporation, left traces of a hygroscopic residue. This residue gave a reddish brown precipitate with Nessler's reagent.

Identification of Sugar As Glucose—After extraction with ether, the alkaline Solution 3 was passed through Amberlite MB-3 and concentrated *in vacuo* to a colorless sirup which was dissolved in 0.5 cc. of water. An aliquot of 0.1 cc. was removed and 0.005 cc. each was used for the following tests: (1) the sulfuric acid test (15), of which the intensity of the pink color formed corresponded to 35 γ of glucose; (2) the glucose oxidase test according to Keston (17), tests with this sensitive specific glucose reagent[10] being strongly positive.

Preparation of Potassium Gluconate—The residual 0.4 cc. of the concentrated Solution 3, which contained 2.6 mg. of glucose as estimated by the sulfuric acid test, was taken to dryness and oxidized according to Moore and Link (18).[11] This yielded 2.7 mg. of a crystalline powder, melting at 178–180° with decomposition which agrees with the melting point reported for potassium gluconate. A mixed melting point with a sample of potassium gluconate gave no depression. The infra-red spectrum of the derivative was found to be exactly identical with that of potassium gluconate.

Test for Volatile Bases—Solution 4, resulting from acid hydrolysis, was made alkaline with 3 cc. of 1 N NaOH and refluxed for 4 hours. A stream of nitrogen which was kept bubbling through the solution was passed through a trap containing 5 cc. of 0.01 N HCl. At the end of the heating period, an aliquot of the trap solution showed no decrease in acidity. After lyophilization of the trap solution, a trace of a hygroscopic residue was left which was insoluble in ether. Addition of a drop of water and Nessler's reagent produced a reddish brown precipitate.

[10] The authors are greatly indebted to Dr. A. S. Keston for a gift of his glucose oxidase reagent.

[11] The derivative was prepared on a micro scale with only approximately 0.001 of the quantities described by Moore and Link. As little as 1 mg. of glucose can be converted into crystalline potassium gluconate with this microprocedure.

Reductive Cleavage with $LiAlH_4$—To an ether solution of 36 mg. of cord factor (PN, DT, C), a solution of $LiAlH_4$ in ether was slowly added. After refluxing for 1 hour, water was added and the ether layer separated. A waxy residue was obtained after evaporation of the ether which was purified by precipitation from ether with methanol, yielding 26 mg. of a white powder, m.p. 46–48°. Found, C 81.87, H 13.51; calculated for α-mycolyl alcohol ($C_{88}H_{178}O_3$), C 82.29, H 13.97. The infra-red spectrum (Fig. 2, Curve 7) was identical with that of a sample of α-mycolyl alcohol obtained after reduction of mycolic acid with $LiAlH_4$ (13).

The water solution was lyophilized after the insoluble hydroxides had been filtered off. The dry residue was then acetylated with acetic anhydride and sodium acetate. The acetylated carbohydrate fragment was isolated in the usual manner by extraction with chloroform, and a few mg. of a sirupy residue were obtained. It did not crystallize. Its infra-red spectrum (Fig. 2, Curve 9) has the characteristic bands of sugar acetates with strong acetyl absorptions at 1750, 1375, 1230, and 1040 cm.$^{-1}$. The lack of amide C=O absorptions around 1650 cm.$^{-1}$ indicates the absence of *N*-acetyl groups.

Derivatives of Cord Factor; Acetylation—It has been reported (19) that acetylation of cord factor with acetic anhydride in the presence of sodium acetate led to a partially acetylated product which was about 10 times less toxic. Asselineau found that a completely acetylated derivative could be obtained by treatment of cord factor with acetic anhydride in pyridine solution.[12] According to his directions, 60 mg. of cord factor were dissolved in 1 cc. of benzene to which 3 cc. of pyridine and 1.5 cc. of acetic anhydride were added. After the solution was allowed to stand for 3 days at room temperature, it was concentrated on a boiling water bath, and the reaction product precipitated with methanol. Purification by precipitation from ether with methanol yielded 40 mg. of a white powder, m.p. 34°. Its infra-red spectrum (Fig. 3, Curve 10) shows strong acetyl bands. At the same time, the strong —OH band at 3350 cm.$^{-1}$ has disappeared, indicating that acetylation was complete. The lack of absorptions in the vicinity of 1650 and 1550 cm.$^{-1}$ indicates that no nitrogen is available for acetylation. Found, C 75.09, H 11.82, N 0.56. Analysis of the parent cord factor preparation gave the following: C 77.24, H 12.16, N 0.65. Calculation of the number of acetyl groups from the difference in the C and H values between the parent compound and the acetyl derivative, a computation which is subject to considerable uncertainty in the case of such a large molecule, gives rise to the estimate of two to five acetyl groups per molecule.

A more accurate method is the direct acetyl determination (20). Sa-

[12] Asselineau, J., personal communication.

ponification of 4.553 mg. of acetyl derivative in 5 cc. of isopropanol containing 100 mg. of KOH for 1 hour gave a volatile acid equivalent of 1.13 cc. of 0.013 N NaOH, or 13.6 per cent $COCH_2$. This corresponds to five to six acetyl groups per molecule, assuming a molecular weight of 1500 to 1600 for cord factor.

Pipsylation (*21*)—48 mg. of cord factor were dissolved in 10 cc. of chloroform containing 0.1 cc. of pyridine and 205 mg. of I^{131}-radioactive *p*-iodobenzene sulfonyl chloride.[13] After the solution was allowed to stand at 25° for 30 minutes, it was concentrated to 5 cc. on a water bath and extracted successively with 1 N NaOH and water until radioactivity measurements indicated that pipsylic acid was no longer being extracted. The

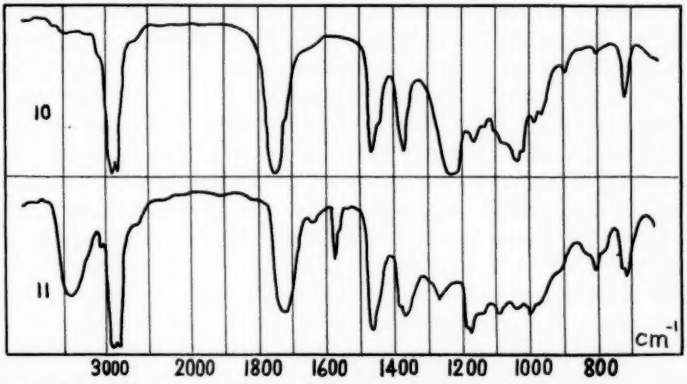

FIG. 3. Infra-red spectra of derivatives of cord factor (taken from melts). Curve 10, acetylated cord factor; Curve 11, pipsylated cord factor.

chloroform layer was dried over Na_2SO_4 and the solvent evaporated. The residue was dissolved in ether, precipitated with methanol, filtered, and washed with methanol. 35 mg. of a white powder, m.p. 36–38°, were recovered. The specific radioactivity of this product corresponded to about half a pipsyl group per cord factor molecule. The infra-red spectrum also suggested that the reaction product was a mixture containing unchanged cord factor. Chromatography of this mixture on magnesium silicate-Celite yielded two fractions: (1) eluted with benzene-ether 1:1 (sharp band), m.p. 33–34°, containing most of the radioactivity; (2) eluted with ether containing 5 per cent methanol, m.p. 38–40°, its weak radioactivity suggesting that it contained a trace of the pipsyl derivative as an impurity.

The infra-red spectrum of the radioactive fraction (1) (Fig. 3, Curve 11)

[13] The radioactive pipsyl reagent was obtained through the courtesy of Dr. A. S. Keston.

shows the characteristic absorptions of covalent sulfonate at 1370 and 1180 and the *p*-substituted phenyl bands at 1575, 815, and 730 cm.$^{-1}$. The intensity of the —OH band is somewhat less than in cord factor but still strong; at the same time its position has shifted from 3350 to 3450 cm.$^{-1}$, indicating less hydrogen bonding. The decrease in OH absorption, as well as the position of the S=O bands, suggests that the derivative is a sulfonate rather than a sulfonamide (in the latter, the S=O bands are usually lower). This is further evidence for the absence of a primary or secondary amine group, since the latter is more reactive than are hydroxyls.

The radioactivity was measured with a Geiger counter with thin end window tubes. The specific activity was determined by adding a volumetrically determined amount of the sample solution on to a filter paper disk placed in a dish. After evaporation of the solvent, the sample was counted. Before the determination of the specific activity of the pipsyl reagent standard, its purity was ascertained by determination of melting point and ultraviolet absorption. The following values were obtained: (*a*) specific activity of pipsyl derivative Fraction 1 = 3280 c.p.m. per mg. and (*b*) specific activity of the pipsyl residue = 22,600 c.p.m. per mg.

Assuming one pipsyl group per cord factor molecule, we obtain M = 1570 for the molecular weight of cord factor (267 times 22,600, divided by M plus 267, equals 3280, 267 being the molecular weight of the pipsyl residue).

This value agrees well with estimations derived from the degradation experiments. The pipsyl derivative was not toxic for mice.

Fraction 2, m.p. 38–40°, which was eluted with ether-methanol, has the typical infra-red spectrum of cord factor and was toxic, thus proving that the unchanged part of the original reaction mixture had been removed during the chromatographic purification of the latter.

DISCUSSION

Although the final elucidation of the chemical structure of cord factor has not yet been accomplished, the identification of mycolic acid and glucose as structural units allows its characterization as a new type of glycolipide which has not been previously isolated from tubercle bacilli.

From the data collected in the experimental part, it is possible to outline broadly the structure of the cord factor molecule. It has been shown that a two-step hydrolysis splits the molecule into at least three fragments according to the following sequence:

$$\text{Cord factor} \xrightarrow{\text{KOH}} \text{mycolic acid (83\%)} + \text{glucoside} \xrightarrow{\text{HCl}} \text{glucose (9\%)} + R$$

It is evident from this that the fragments are connected with each other by two different types of linkages, one broken by the action of alkali and

$LiAlH_4$ and the other by acid. The former is an ester linkage involving the carboxyl group of mycolic acid, since it is split by saponification as well as reduction, liberating mycolic acid or mycolic alcohol, respectively.

The acid-sensitive linkage must be of glucosidic nature because its cleavage gives rise to a reducing sugar identified as glucose. The fact that it is stable towards alkali is in accordance with this conclusion.

Whether the third fragment, *i.e.* the still unknown residue *R*, is attached to C_1 of glucose through oxygen or nitrogen cannot be decided. The possibility of an *N*-glucoside is conceivable since the glucosidic fragment *R* most probably contains the 1 atom of nitrogen found in the cord factor molecule. However, from what is known about *N*-glucosides, they are apparently rather unstable unless the nitrogen, being a member of an unsaturated ring system, is stabilized by resonance (22). The possibility of the presence of an unsaturated ring structure, however, seems unlikely because of the lack of the corresponding absorptions in the ultraviolet as well as in the infra-red region of the spectrum.

Also it is questionable whether the mycolic acid residue is attached to one of the hydroxyls of glucose or to the aglycon *R*: mycolic acid-glucose-*R*; mycolic acid-*R*-glucose. Attempts to decide this question by direct acid hydrolysis of cord factor failed because of incomplete reaction due to the great insolubility of the material in aqueous solvents. Since in these experiments both glucose and mycolic acid were liberated in small amounts, it is obvious that conditions permitting the selective hydrolysis of the glucoside linkage have not yet been found.

The fragment which is linked to C_1 of glucose has not yet been isolated. Information about its nature can be deduced only by inference. If the molecular weight of cord factor is known, the size of the unknown fragment can be estimated by difference. The molecular weight of cord factor was determined by three independent methods: (1) pipsylation, (2) calculation based on recovery (83 per cent) and titration equivalent (1300) of mycolic acid, and (3) calculation based on C, H distribution in mycolic acid and cord factor. All three values were in close agreement, corresponding to 1570 ± 25. Since the total recovery of mycolic acid and glucose was 92 per cent, the unknown fragment cannot constitute more than 8 per cent, corresponding to a molecular weight of 100 to 150. It is likely to contain the $N—CH_3$ group found in cord factor. The infra-red spectra of the acetyl derivatives fail to show any *N*-acetyl absorptions. Infra-red spectroscopy also indicated that the non-reducing glucosidic fragment contains no C=O function (acid, ester, keto, amide). The relative intensity of the CH_2 bands, on the other hand, is stronger than in glucose. All these facts together suggest that the unknown fragment is a non-volatile aliphatic trisubstituted amine, possibly containing alcoholic —OH.

Glucose has been obtained from tubercle bacilli only in the form of the

non-reducing disaccharide trehalose. Anderson has shown that trehalose is the structural unit of a polysaccharide extracted from tubercle bacilli (23), but he also found it as a water-soluble component after alkaline hydrolysis of the acetone-soluble neutral fat (24). He pointed out the peculiarity that acid-fast bacteria grown on a glycerol-containing medium synthesize a disaccharide such as trehalose with which the fatty acids are combined to form neutral fat. Although cord factor appears to be another example of this type of structure, the difference between the two compounds is nevertheless striking. Instead of the relatively low molecular fatty acids of the acetone-soluble fat, cord factor contains high molecular mycolic acid as the lipide component.

The relationship between the lipopolysaccharide (Wax D (25)) or the similar "Pmko" of Choucroun (26) and cord factor has been discussed in a previous paper (3). Both cord factor and Wax D are carbohydrate- and nitrogen-containing esters of mycolic acid, and both are components of Anderson's purified wax fraction, but, according to Anderson and Lederer,[14] the polysaccharide part of Wax D is composed of galactose, arabinose, and glucosamine, whereas the glucose content of cord factor makes this compound a chemically distinct constituent of the purified wax of the tubercle bacillus.

SUMMARY

The isolation of a toxic glycolipide "cord factor" from various strains of virulent tubercle bacilli is described. It was found to be a characteristic component of the Wax C fraction of the purified wax.

Chemical studies show that cord factor is an ester of mycolic acid having a molecular weight of about 1570. Alkaline hydrolysis splits the ester linkage, liberating 1 molecule of mycolic acid and 1 molecule of a non-reducing glucoside. Subsequent acid hydrolysis of the latter cleaves the glucosidic linkage, yielding glucose and a non-volatile aliphatic fragment which has not yet been identified. Chemical data suggest that the unknown aglycon contains 1 atom of nitrogen which carries a methyl group and cannot be acetylated.

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AMINE OXIDASES

XI. INHIBITION OF MONOAMINE OXIDASE BY 1-ISONICOTINYL-2-ISOPROPYLHYDRAZINE*†

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Until recently, no specific and potent inhibitor of monoamine oxidase (MO) has been known. However, in 1-isonicotinyl-2-isopropylhydrazine (IIH) an agent was found which blocks this enzyme very effectively (2, 3). Since this substance seems to be of great help in the study of the reaction mechanism of MO and of the largely unknown metabolic function of this enzyme, it was considered necessary to analyze the effect of IIH on MO further. Some of the results obtained with various enzyme preparations from a number of organs and from different species are presented in this paper. In a subsequent publication, the specificity and the mode of action of IIH will be discussed.

Materials and Methods

Tissues were forced through an Altmann press, and the pulp was homogenized in from 4 to 9 volumes of either 0.25 M or 0.88 M sucrose. Homogenization was carried out in glass vessels with a motor-driven glass or Teflon pestle. Mitochondria were isolated by differential centrifugation according to the method of Hogeboom, Schneider, and Palade (4). Morphology and staining characteristics of the preparations were examined with the phase microscope and with Janus green B. The succinoxidase activity (manometrically determined) (5) of hog liver mitochondrial preparations represented 82 per cent of original tissue activity. This same fraction contained 45 to 50 per cent of the MO activity of homogenates.

Another fraction (microsomes) containing 8 to 23 per cent of the MO activity was obtained when, after isolation of the mitochondria from 0.25 M sucrose suspension, the supernatant solution was centrifuged at 105,000 $\times g$ for 90 minutes in the Spinco ultracentrifuge. Hog liver mitochondria

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† Amine oxidases, X (1).

XUM

were disrupted by the combined effects of 9 kc. sonic oscillations and the use of deoxycholic acid (6).[1] Tissue slices were prepared by the technique of Deutsch (7).

In experiments on intact animals, adult rats (Sprague-Dawley), guinea pigs, cats, and dogs of both sexes were used. Cats and dogs were under pentobarbital anesthesia.

TABLE I

Inhibition of Liver and Brain MO by 1×10^{-4} M IIH

The reaction vessels contain the equivalent of 0.1 gm. of fresh tissue (liver homogenate) or 0.2 gm. (liver mitochondria, brain homogenate) or 0.33 gm. (brain mitochondria). Variation of results is indicated by standard deviations and by the number of independent determinations. The conditions of measurement and presentation of results are those described as "standard." $Q_{ox.}$ = microatoms of oxygen per hour per gm. of tissue; $Q_{am.}$ = ammonia liberated, in micromoles per hour per gm.

Tissue and preparation	Oxygen uptake		Ammonia production	
	$Q_{ox.}$	Inhibition	$Q_{am.}$	Inhibition
		per cent		*per cent*
Rat liver homogenate	123 ± 32 (7)*	13 (3)	59 ± 5 (6)	18 (3)
" " mitochondria	66 ± 24 (7)	59 (4)	25 ± 5 (6)	66 (3)
" brain cortex homogenate	29 ± 8 (6)	46 (2)	27 ± 1.4 (6)	49 (2)
" " " mitochondria	14 ± 5 (6)	67 (3)	13 ± 4 (6)	62 (3)
Guinea pig liver homogenate	162 ± 23 (6)	23 (4)	57 ± 9 (5)	0 (3)
" " " mitochondria	80 ± 12 (6)	79 (7)†	26 ± 3 (8)	65 (6)‡
" " brain cortex mitochondria	6 ± 3 (6)	69 (4)	3.4 ± 1.2 (6)	72 (4)

* Number of independent determinations.
† $Q_{ox.} = 17 \pm 12$.
‡ $Q_{am.} = 9 \pm 6$.

Substrates and inhibitors obtained from commercial sources were employed without further purification except for tyramine hydrochloride, which was recrystallized when necessary.[2]

Our standard procedure for the measurement of MO activity and presentation of results was as follows: The enzymic system was incubated at 38° in 2 ml. of M/15 phosphate buffer, pH 7.2, in an atmosphere of oxygen.

[1] A detailed description of the liberation of MO from mitochondria will be given in a subsequent paper.

[2] We wish to extend our thanks to Dr. M. J. Schiffrin, Hoffmann-La Roche, Inc., for a supply of marsilid (1-isonicotinyl-2-isopropylhydrazine phosphate = iproniazid = IIH), to Dr. M. L. Tainter, Sterling-Winthrop Research Institute, for a sample of *l*-epinephrine bitartrate, and to Dr. R. K. Richards, Abbott Laboratories, for serotonin creatinine sulfate.

The substrate, 0.01 M tyramine hydrochloride, was added at least 10 minutes after IIH (10^{-4} M). Mitochondria, unless stated otherwise, were prepared with 0.88 M sucrose solution. From the curve of oxygen uptake as a function of time, the initial velocity of oxygen consumption was derived and given in terms of microatoms of oxygen per hour per gm. of fresh tissue ($Q_{ox.}$). At the end of 2 hours of incubation, an aliquot of the reaction mixture was transferred into Conway units; the amount of liberated ammonia was expressed as micromoles per hour per gm. ($Q_{am.}$).

Concentrations were calculated for the final volume of the test solution. For actual values, blank determinations were subtracted from those for the complete reaction systems.

TABLE II

Effect of IIH on MO of Various Hog Liver Preparations

The reaction vessels contained the equivalent of fresh tissue listed in the third column. Exception was made for Experiment 7, in which the number of mg. of nitrogen are listed; the Q values, therefore, represent in this case the number of microatoms or micromoles per mg. of nitrogen. In Experiment 5, the enzyme was prepared from fresh mitochondria, in Experiment 6 from mitochondria previously treated with acetone, and in Experiment 7 by fractionation with ammonium sulfate. The duration of the experiments was 1 hour except for Experiment 1 (2 hours) and Experiment 6 (1½ hours). The volume of the systems was 2 ml., except for Experiments 5 and 7 (3 ml.). The medium of Experiment 2 contained 0.13 M sucrose and 0.04 M phosphate. The other conditions of measurement and presentation of results are those described as "standard."

Experiment No.	Preparation	Weight	Oxygen uptake			Ammonia production		
			$Q_{ox.}$	Inhibition by IIH		$Q_{am.}$	Inhibition by IIH	
				1×10^{-4} M	1×10^{-3} M		1×10^{-4} M	1×10^{-3} M
		gm.		*per cent*	*per cent*		*per cent*	*per cent*
1	Slices	0.1	137	59	85	69	36	90
2	Homogenate	0.25	253	15	51	62	3	40
3	Mitochondria	0.35	114	100	100	38	100	100
4	Microsomes	0.49	21	48	48	18	100	100
5	Free enzyme	0.77	32	31	90	17	42	94
6	" "	0.24	24	100	100	22	100	100
7	" "	0.46	15	100	100	10	100	100

Results

Effect of IIH on MO of Various Species—Results obtained with rat and guinea pig preparations are summarized in Table I. MO of mitochondria prepared from hog kidney cortex and from liver or brain of mouse, rabbit,

cat, dog, hog, cattle, and man were inhibited from 90 to 100 per cent by 10^{-4} M IIH.

Sensitivity of Various Preparations of MO to IIH—Marked differences in the degree of inhibition were observed when MO was prepared in various ways (Table II). Similar results were found with rat liver MO.

Inhibition of MO in Vivo—The application to dogs of 4×10^{-4} mole of IIH per kilo of body weight caused a total inactivation of MO in liver homogenate and 73 per cent reduction in activity of brain homogenate.

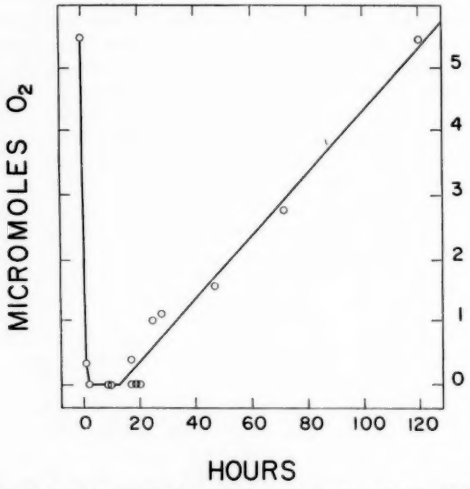

FIG. 1. Inactivation of rat liver MO by IIH. 5×10^{-5} mole of IIH per kilo of body weight were injected subcutaneously at zero time; the abscissas, therefore, indicate time between injection and sacrifice of the animals. Conditions of measurement of MO activity of homogenate are those described as "standard." The ascending curve was determined by the method of least squares.

A study of restoration of MO activity in intact rats as a function of time revealed that approximately 5 days were required for recovery of normal activities of liver homogenate (Fig. 1) and of liver and brain mitochondria. The reappearance of MO might not have been due to a reactivation of the original enzyme molecule, but to the formation of new MO.

Effect of IIH on Degradation of MO Substrates Other Than Tyramine—Tyramine was replaced as a substrate by tryptamine, 5-hydroxytryptamine (serotonin), epinephrine (Table III), butylamine, and amylamine. Under standard conditions, the same sensitivity of MO of liver mitochondria (cattle, rabbit) towards 10^{-4} M IIH was recorded.

Reversibility of MO Inhibition by IIH—After having inactivated MO

in vitro with IIH, attempts to restore the activity of MO by dialysis were unsuccessful. Similar negative findings were observed when liver mito-

TABLE III

Inhibition of Degradation of Tryptamine, 5-Hydroxytryptamine, and Epinephrine by IIH

The reaction vessels contained mitochondria equivalent to 0.2 gm. of beef liver; substrates, 0.01 M; the conditions of measurement and presentation of results are those described as "standard;" epinephrine determination was carried out in the presence of 1×10^{-3} M cyanide and 2 mg. of glutathione.

Substrates	Oxygen uptake			Ammonia production		
	$Q_{ox.}$	Inhibition by IIH		$Q_{am.}$	Inhibition by IIH	
		1×10^{-4} M	1×10^{-3} M		1×10^{-4} M	1×10^{-2} M
		per cent	*per cent*		*per cent*	*per cent*
Tryptamine	58	99	100	18	100	97
5-Hydroxytryptamine	41	92	86	13	95	94
l-Epinephrine bitartrate	7	100	100	6	100	100

TABLE IV

Effect of Substrate Concentration on Inhibition of MO by IIH

The enzyme was prepared from hog liver mitochondria by ammonium sulfate fractionation; tyramine and inhibitor were added either simultaneously or MO was pretreated with IIH at 38° for 30 minutes before the substrate was tipped into the enzyme solution. The conditions of measurement are those described as "standard," with the exception of the incubation time (1 hour). Ammonia produced per vessel per hour is reported. The percentage inhibition, calculated on the basis of the initial rate of oxygen uptake, was almost identical with the values for ammonia production.

Concentration of tyramine	NH_3 produced without inhibitor	Inhibition	
		1×10^{-4} M IIH added simultaneously	1×10^{-5} M IIH preincubated
M	*10^{-6} mole*	*per cent*	*per cent*
0.05	3.6	1	84
0.01	4.3	47	88
0.002	3.5	69	80

chondrial preparations, isolated from IIH-treated rats (6×10^{-5} mole per kilo), were subjected to dialysis in a constant flow apparatus. Even the exhaustive dialysis of a soluble MO, obtained from hog liver mitochondria, reduced the degree of inhibition only from 77 to 54 per cent.

Protective Action of Substrate—The degree of inhibition of mitochondrial MO activity depended on whether IIH was added to the system prior to or

simultaneously with tyramine. In the former case, the substrate concentration did not notably influence the inhibitory response, while, in the latter instance, the inhibition decreased with increasing tyramine concentration (Table IV).

In other experiments, preparations of free and mitochondrial MO were incubated with IIH for various periods before the substrate was added. The extent of inhibition increased with an increasing period of incubation. In general, the maximum was reached between 6 and 12 minutes (Fig. 2).

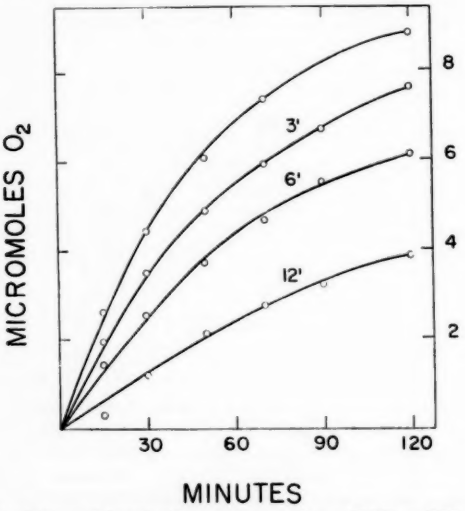

FIG. 2. Effect of time of incubation on the action of IIH on MO. Each Warburg vessel contained the amount of mitochondria equivalent to 0.2 gm. of guinea pig liver. IIH (5×10^{-5} M) was tipped in from one side arm at successive intervals of 3, 6, and 12 minutes ahead of tyramine, which was added from a second side arm. Conditions of measurement are those described as "standard."

DISCUSSION

All results presented in this paper indicate that 1-isonicotinyl-2-isopropylhydrazine (IIH) acts directly on MO. Although tyramine protects the enzyme partially against inhibition, the interaction between enzyme and inhibitor is not based on simple competitive inhibition. Even in the absence of substrate, some minutes were required to produce maximal inactivation. Furthermore, the blocking caused by IIH cannot be substantially reversed as yet.

The only preparations which are relatively unaffected by IIH are homogenates. This insensitivity apparently is due to agents present in the cel-

lular fraction obtained after the removal of the particulate matter by centrifugation of homogenate for 60 minutes at 150,000 × *g*, since the addition of this fraction to mitochondrial MO reduces its sensitivity towards IIH.[3] *In vivo* these agents do not seem to come into play, as shown by the efficiency of IIH on MO of the intact animal.

The changes wrought by IIH on MO can easily be detected *in vivo*. The pharmacological response to several sympatheticomimetic amines is potentiated by previous administration of IIH, in exact agreement with enzymological data (8, 9). The same is true for the changes produced by IIH on the metabolism of epinephrine (10). Finally, IIH has become a valuable aid in the histochemical determination of MO (11). Thus, IIH turned out to be a promising tool for the analysis of the metabolic function of MO and is used for this purpose in this and several other laboratories.

There have been few reports published concerning the effect of IIH on enzymes. Diamine oxidase (2), spermine oxidase (12), and amphetamine deaminase (13)[4] are much less sensitive to IIH than MO. Bacterial catalase is only slightly susceptible to 0.03 M IIH (14), and beef spleen DPNase is 50 per cent inhibited by 0.002 M IIH (15).

SUMMARY

1. Monoamine oxidase (MO) of mitochondrial preparations of various organs and several species is strongly inhibited by 10^{-4} M 1-isonicotinyl-2-isopropylhydrazine (IIH).

2. MO of tissue homogenates is considerably less sensitive than soluble MO and MO of mitochondria, microsomes, and tissue slices.

3. On administration to rats, IIH inactivates MO *in vivo*. The restoration of enzyme activity takes about 5 days.

4. Preparations of IIH-inactivated MO cannot be reactivated by dialysis.

5. It takes 6 to 12 minutes of incubation of enzyme with IIH to produce maximal inhibition. Tyramine protects MO against the action of IIH. This protection becomes more effective with increasing tyramine concentration when substrate and inhibitor are added simultaneously to MO.

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LIGHT SCATTERING OF D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*

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The properties and structure of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase (1) are of special interest and importance, since this enzyme is a readily available crystalline pyridino protein with a firmly bound coenzyme. Evidence has been presented recently (2, 3) that, as isolated from skeletal muscle, the enzyme is combined with 2 moles of DPN[1] per mole of protein. It is inherent in the concepts of molecular structure that an enzyme molecule capable of binding a coenzyme must possess an integral number of coenzyme binding sites. If a particular protein preparation with an enzymatic function (and homogeneous by the usual standards) is found experimentally to have an integral number of such sites per mole, this fact may be regarded as indicative, although not conclusive, evidence that the protein molecule and the enzyme molecule are identical. The application of this criterion obviously requires accurate data not only on the maximal coenzyme-binding capacity per unit mass, but also on the protein molecular weight as well.

The only reported molecular weight for glyceraldehyde phosphate dehydrogenase was obtained by Taylor (4) using sedimentation and diffusion. In view of the importance of the conclusions based upon this constant, it seems desirable to redetermine it by a second independent method.

EXPERIMENTAL

Preparation of Enzyme—The enzyme was prepared from rabbit skeletal muscle by the method of Cori, Slein, and Cori (1), except that 0.002 M EDTA (disodium salt) was added (5) to all reagents used in the preparation. The enzyme was recrystallized from four to eight times before use and stored near 0° as a crystal suspension in the mother liquor. Enzyme prepared in the absence of EDTA was found to lose enzymatic activity rapidly at room temperature and to be unstable in light scattering experiments, whereas that prepared in the presence of EDTA was very stable.

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[1] The following abbreviations are used: DPN for diphosphopyridine nucleotide; EDTA for ethylenediaminetetraacetic acid.

The addition of EDTA was kindly suggested by Professor E. Racker who previously observed the stabilizing effect. Before use, the crystals were centrifuged and dissolved in and equilibrated with the appropriate buffer.

Activity Measurements—Enzymatic activity was assayed by the procedure of Cori *et al.* (1) with glyceraldehyde-3-phosphate as a substrate and by the method of Warburg and Christian (6) and of Velick (7) with glyceraldehyde as a substrate.

DPN Assay—The enzyme was assayed for DPN by enzymatic reduction according to Taylor *et al.* (2), except that glyceraldehyde was used as the substrate. A typical reaction mixture contained sodium pyrophosphate 0.03 M, cysteine 0.004 M, sodium arsenate 0.0062 M, DL-glyceraldehyde 0.029 M, and enzyme 5 mg. per ml., the final pH being 8.3. The molar extinction coefficient of reduced DPN at 340 mμ was taken to be 6250 from Ohlmeyer (8) and Horecker and Kornberg (9). It may be pointed out that no assumption has been made here that this method gives an accurate measure of the total DPN content of the enzyme, the results of the assay being chiefly of interest for comparison with previous work (3).

Absorption Measurements—Measurements of light absorption were made in a Beckman DU spectrophotometer with 1 cm. quartz cells. The wavelength scale was checked at the 4358 and 5461 A mercury lines. The photometric scale was calibrated at several points with alkaline potassium chromate solutions from the absorption data of Haupt (10).

Protein Concentration—Protein concentration was measured routinely by absorption at 280 mμ. The value used for the absorptivity of the enzyme was based upon a dry weight measurement on electrodialyzed solutions. The solutions were evaporated at about 70° overnight in a hot air oven and the residues dried to constant weight at 100° *in vacuo* over phosphorus pentoxide.

Refractive Index Increment—Refractive indices were measured in a Phoenix differential refractometer which had been calibrated with potassium chloride, from the data of Kruis (11) (cited by Stamm (12)). The protein solutions were electrodialyzed and measured *versus* water.

Electrodialysis—A Pauli type apparatus of 15 ml. capacity was used. After an ordinary dialysis against distilled water the solutions were electrodialyzed until the conductivity of the protein solution was nearly constant (~48 hours).

Light Scattering Measurements. Apparatus—Measurements of scattered intensity were carried out with an instrument[2] described by Brice *et al.* (13) with very minor modifications. The stability of the instrument was increased somewhat by using a General Electric A-H4 mercury arc as the

[2] Purchased from the Phoenix Precision Instrument Company, Philadelphia, Pennsylvania.

light source. A precision helical potentiometer was placed in the galvanometer circuit to permit the galvanometer to be used at constant deflection. The potentiometer itself furnishes the damping resistance for the galvanometer. The solution cell for scattering measurements was of square cross-section (1 cm. × 1 cm.) and required about 3.5 ml. of solution.

Calibration—The results were reduced to an absolute scale by two methods. In the first, intensities were compared with the scattering from a standard sample of Dow Styron (polystyrene), kindly furnished by Dr. P. Debye and Dr. A. M. Bueche of Cornell University. The reduced intensity for a 0.75 per cent solution of this standard in butanone was taken from the measurements of Carr and Zimm (14).

In the second method a sample of colloidal silica (du Pont Ludox) was diluted to approximately 0.6 per cent in 0.2 M NaCl and centrifuged at 5000 × g for 3 hours. The transmission of the supernatant fluid was measured in a Beckman DU spectrophotometer with 10 cm. cells and a mercury arc source. Both the angular dependence and polarization of a silica sol prepared in this way are very small, and hence the reduced intensities are readily calculated from the measured turbidity. This method of calibration has been used by Edelhoch *et al.* (15), by Mommaerts (16), and by Oth *et al.* (17). The results of the two calibrations, differing by about 2 per cent, were averaged in computing the instrument constant.

Neutral filters used in the light scattering measurements were calibrated by transmission measurements both in a Beckman DU spectrophotometer and in the light scattering instrument. These two methods agreed to within 1 per cent.

Correction Factors—The correction factors applied to the light scattering measurements deserve special mention.

1. Correction for turbidities measured in a spectrophotometer. Since not only transmitted light but also some scattered light enters the photometer, it is necessary to make an extrapolation with diaphragms of successively smaller size over the phototube. For our instrument the turbidity (for a silica sol in a 10 cm. cell) obtained by extrapolation is 1.03 times as great as the value obtained with no diaphragm.

2. Refractive index and volume corrections (C_n and C_v). The corrections derived by Carr and Zimm (14) were used throughout.

3. Refractive index correction for the transmitted intensity. The necessary correction is easily obtained experimentally by comparing transmitted intensities at constant incident intensity for liquids of different refractive indices. In comparing dilute aqueous solutions with the polystyrene standard in butanone, it was found that a factor of 1.01 should be introduced.

4. Attenuation of the transmitted beam by scattering. For the rather

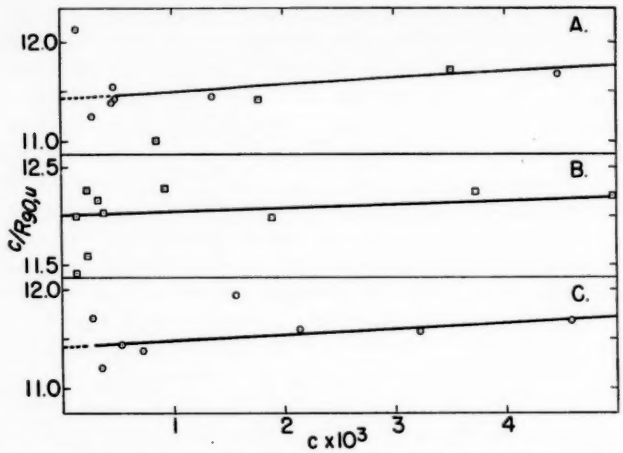

FIG. 1. Light scattering of D-glyceraldehyde-3-phosphate dehydrogenase at 436 mμ. $R_{90,u}$ is the reduced scattered intensity (cm.$^{-1}$), while c is the protein concentration in gm. per ml. Curve A, in potassium phosphate buffer, pH 6.55, $\Gamma/2$ = 0.10; Curve B, in potassium phosphate buffer, pH 7.6, $\Gamma/2$ = 0.135; Curve C, in 0.15 M sodium chloride at pH 7.3. In each case circles indicate that 0.002 M EDTA was present in addition to the other electrolytes; squares, no EDTA.

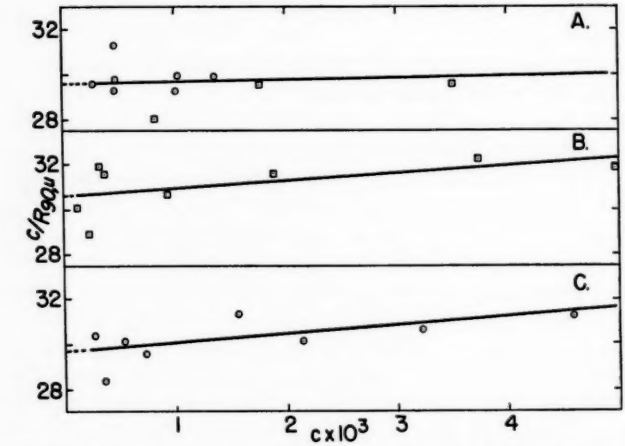

FIG. 2. Light scattering of D-glyceraldehyde-3-phosphate dehydrogenase at 546 mμ; symbols as in Fig. 1.

high turbidities of the silica or polystyrene solutions it cannot be assumed that the transmitted intensity is equal to the incident intensity. The square cell used in this case compensates at least partly for this factor, and no correction was made.

5. Sensitivity of the photomultiplier tube to the state of polarization. The 1P21 tube used was found to respond equally (±1 per cent) to the horizontal and vertical components, and no correction was necessary.

Preparation of Solutions—Solutions of the enzyme in the appropriate buffer or salt solution were centrifuged either at 20,000 × g or at 80,000 × g for 2 to 4 hours. The supernatant fluid was then drawn off for light scattering measurements. The intensities from solutions centrifuged at

TABLE I

Light Scattering of Glyceraldehyde Phosphate Dehydrogenase

Solvent	λ_0	$(c/R_{90,u})_{c=0}$*	$M \times 10^{-5}$
	A		
Potassium phosphate, pH 6.55; Γ/2 = 0.10	4358	11.4	1.46
	5461	29.6	1.48
" " " 7.6; Γ/2 = 0.135	4358	12.0	1.39
	5461	30.6	1.43
Sodium chloride, 0.15 M; pH 7.3	4358	11.4	1.46
	5461	29.7	1.47

For the calculations above the following values were taken for other necessary constants: depolarization $(\rho_{90,u}) = 0.01$; $dn/dc = 0.190$ at 4358 A and 0.185 at 5461 A.

* c in gm. per ml.

the higher speed were found to be as much as 5 per cent below those centrifuged at 20,000 × g. All glassware used in handling the centrifuged solutions was washed in dust-free water from an enclosed Pyrex still and dried either in an oven or by acetone vapor. Measurements were made only on solutions found to be free of dust when viewed at low scattering angles.

Results

Data from the light scattering experiments are shown in Figs. 1 and 2. The extrapolated values of $(c/R_{90,u})$ are given in Table I together with molecular weights calculated from Equation 1, after correction for depolarization.

The ultraviolet extinction coefficient at 280 mμ, *i.e.* $1/lc \log (I_0/I)$, was found to be 1000, where l is the path length in cm. and c the concentration in gm. per ml.; Velick's value (3) for the same constant is 1017. The re-

fractive index increment for the enzyme was found to be 0.190 at 4358 A and 0.185 at 5461 A with concentration in gm. per ml. Duplicate determinations of the DPN content of the enzyme were in good agreement and indicated that 58,500 gm. of enzyme contain 1 mole of DPN. This agrees closely with the value given by Velick *et al.* (3). The preparations used in this work were found to have enzymatic activities ranging up to about 2.5 times those reported by Cori *et al.* (1).

DISCUSSION

Molecular weights from light scattering can be found from Equation 1 for two-component systems.

$$\left[\frac{R_{90,u}}{c}\right]_{c=0} = \frac{KM}{2} \tag{1}$$

$R_{90,u}$ is the reduced excess scattered intensity at 90° with unpolarized incident light; M is the molecular weight of solute present at concentration c (expressed here in gm. per ml.), and $K = 4\pi^2 n^2 (dn/dc)^2/\lambda_0^4 N_0$ where n is the refractive index of the solvent, dn/dc the refractive index increment, λ_0 the wave-length *in vacuo*, and N_0 Avogadro's number. When three or more components are present, the light scattering equation takes the form (18)

$$R_{90,u} = \frac{K'' \sum_i \sum_j \Psi_i \Psi_j A_{ij}}{2 \mid a_{ij} \mid} \tag{2}$$

where $K'' = 1000K/((dn/dc)^2)$. In Equation 2 the determinant $\mid a_{ij} \mid$ consists of terms $a_{ij} = \partial \ln a_i/\partial m_j$, where a_i is the activity of component i and m_j is the molarity of component j. In the double summation extending over all but one of the components, the term A_{ij} denotes the cofactor of the term a_{ij} in the determinant $\mid a_{ij} \mid$; that is, the determinant obtained from $\mid a_{ij} \mid$ by striking out the row and column in which a_{ij} appears and multiplying the resulting determinant by $+1$ if $i + j$ is even and by -1 if $i + j$ is odd. Ψ stands for the molar refractive increment. Equation 2 reduces to Equation 1 for two components.

The systems actually studied contained four components. To calculate the additional terms in Equation 2 requires knowledge of the coefficients a_{ij}; in the case of Curve A in Fig. 1, for example, one needs data on the change in activity of protein with changing concentration of dipotassium hydrogen phosphate, at constant protein concentration. Fragmentary data of this sort are available from equilibrium dialysis experiments (19), but are not sufficiently extensive to be applied to the problem at hand.

The system bovine serum albumin, sodium chloride, and water has been thoroughly investigated by Edsall *et al.* (20). Only one term in Equation

2 was found to be significant; hence Equation 1 describes the scattering satisfactorily. In the present case it is not certain that the same approximation can be made, since phosphate is known to be bound strongly by the enzyme (19). However, the data in sodium chloride (Figs. 1 and 2 and Table I) are very similar to those in phosphate, and it seems likely that the contributions of the phosphate-protein interaction to the scattering can be neglected. It may be noted from Table I that the results obtained in potassium phosphate at pH 7.6 give the lowest values for M. These solutions were clarified at 80,000 $\times$ g and probably represent the most reliable values. Data in sodium chloride and in phosphate of pH

TABLE II

Molecular Weights of Proteins by Different Methods

Protein	$M \times 10^{-3}$*			
	Sedimentation and diffusion	X-ray	Osmotic pressure	Light scattering
Lysozyme	14.7 (21)	13.9 (22)	17.5 (23) 16.6 (21)	14.8 (24)
Lactoglobulin	35.4 (25)	35.4 (26)	35.0 (27)	35.7 (24)
Ovalbumin	49.0 (28)	37.5 (29)	44.9– 45.1 (30) 45.2 (31) 46.0 (32)	45.7 (24)
Bovine serum albumin	68.0 (33)† 65.4 (35)		70.0 (34) 69.0 (36)	75.0 (20) 98.0 (24) 68.0 (15) 69.8 (37)

* The figures in parentheses denote bibliographic references.

† Unpublished data of J. L. Oncley cited by E. J. Cohn (33).

6.55 were obtained on solutions centrifuged at 20,000 $\times$ g. At the higher speeds an appreciable fraction of the protein in solution undergoes true sedimentation and results in the formation of a transparent, gelatinous pellet on the bottom of the centrifuge tube. This pellet serves to entrap particles of dust or other impurities and facilitates the withdrawal of an optically clean sample of solution. Still larger fields would be expected to result only in further sedimentation of the protein without changing the light scattering results.

An estimate of the reliability of light scattering as a means of determining protein molecular weights can perhaps best be arrived at by a comparison of light scattering results with those of other methods such as sedimentation and diffusion, osmotic pressure, and x-ray diffraction. Molecular weight values for several proteins studied by these methods are

summarized in Table II. It appears from these data that the molecular weights from light scattering agree quite well with those of other established methods. In the particular case of bovine serum albumin, recent work (37, 15) has shown that preparations of this protein may be contaminated with high molecular weight impurities, which probably explains the high and variable results obtained by earlier measurements (24).

The data presented here indicate that the molecular weight of muscle glyceraldehyde phosphate dehydrogenase is about 140,000. Taylor (4) reported a value of 118,000 from sedimentation and diffusion, which agrees very well with the 2 DPN molecules found by enzymatic assay. Since it is possible that the molecular properties of the enzyme prepared with and without EDTA are different, we are investigating the sedimentation and diffusion behavior of our preparations.

SUMMARY

A light scattering investigation of D-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle has been made in three different supporting electrolytes and at two wave-lengths. The curves of $R_{90,u}/c$ *versus* c extrapolate with only a small concentration dependence to give a molecular weight not lower than about 140,000.

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THE MODE OF INACTIVATION OF INSULIN BY RAT LIVER EXTRACTS*

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Studies with insulin-I^{131} *in vivo* have suggested that insulin is rapidly cleaved after intravenous injection into rats (3–5). This apparent degradation was found to be significantly decreased after hepatectomy (3), an observation consistent with the finding that the liver is capable of inactivating or removing insulin *in vivo* (6).

In 1929, Shmidt and Saatchian reported that inactivation of insulin by rat tissue extracts occurred to the greatest extent with extracts of the liver (7). Their finding was confirmed by Mirsky and Broh-Kahn, who carried out extensive studies with the liver extract system at pH 7.5 (8). These workers reported that denatured hemoglobin was not proteolytically degraded by this insulin-inactivating system and that soy bean inhibitor and human serum were not inhibitory to the inactivation of insulin. They therefore suggested the possibility of a non-proteolytic inactivation of insulin. This question of proteolysis was reinvestigated by De Barbieri and Grassi (9), who found that denatured globin and peptic hydrolysates of globin were degraded by rat liver extract at pH 7.5 and 3.5. They suggested that, since proteolytic activity was demonstrable, insulin was probably proteolytically inactivated.

In order to try to obtain a definitive answer to this question, a program was initiated to investigate the mode of insulin inactivation by rat liver extract. In this study, evidence was obtained which indicates that insulin is probably proteolytically inactivated by rat liver extract.

Preparation of Extract—This was essentially the procedure of Mirsky and Broh-Kahn (8). Sprague-Dawley rats weighing 200 to 300 gm. were used without fasting. They were decapitated, and the liver was excised and placed in ice-cold water. In the cold room, the liver was homogenized for 2 minutes with water or 0.067 M phosphate buffer, pH 7.5. After cen-

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trifugation for 10 minutes at 10,000 × g, the extract was filtered through gauze and kept on ice. All extracts were prepared immediately before use.

Substrate—This usually consisted of a small amount of insulin-I^{131} solution,[1] with amorphous insulin as additional carrier. The substrate was brought to the desired volume with water or 0.067 M phosphate buffer, pH 7.5. Generally, the latter solvent was used except in studies in which close reproduction of the procedure of earlier workers (8) was desired. At concentrations not readily soluble in these solvents, amorphous insulin was first dissolved in a few drops of 0.01 N HCl. Before use, each aliquot of insulin-I^{131} solution was dialyzed overnight at 5° against 0.067 M phosphate buffer, pH 7.5. Insulin-I^{131} solution, when used without additional carrier, was simply diluted with the appropriate solvent after dialysis.

Incubation—This was performed in a Dubnoff incubator at 37°. The mixture was shaken at a rate of 65 cycles per minute. Incubation was terminated by pouring the mixture into an equal volume of 10 per cent trichloroacetic acid (TCA) in a 15 ml. centrifuge tube. The beaker used in the incubation was rinsed with water, and the contents of the centrifuge tube were mixed thoroughly. After standing for 30 minutes, the material was centrifuged. The precipitate was then washed twice with 5 per cent TCA.

Assays—The amount of radioactivity in the precipitate and combined supernatant fluid was determined with a well type γ counter. Before counting, the precipitate was dissolved in 30 per cent KOH and diluted to a volume equal to that of the supernatant solution. The extent of degradation was expressed either as the percentage of radioactivity in the supernatant fluid or the counts per minute in the supernatant fluid. The control value for the small amount of non-precipitable radioactivity in the insulin-I^{131} was determined by the TCA precipitation of insulin-I^{131} in a 2 per cent solution of dried human plasma. After a 12 hour dialysis of insulin-I^{131}, this correction value was less than 1 per cent of the total radioactivity.

In addition to the radioactive assay, two other assays were used. In some experiments, the supernatant solution was assayed for nitrogen by a semimicro-Kjeldahl method. In others, an aliquot of the incubation mixture was tested for biological activity of insulin according to the rabbit hypoglycemia method of Mirsky and Broh-Kahn (8).

[1] Proteins were iodinated by the Abbott Laboratories. Crystalline zinc insulin, assaying 26 units per mg., was a gift of Eli Lilly and Company. After the iodination of this insulin, analysis indicated that the resultant insulin-I^{131} solution contained carrier, since there were more than 2 insulin molecules for each iodine atom. Of these iodine atoms, no more than 1 in 500 was believed to be radioactive, since iodination was carried out in this ratio of I^{131}:I^{127}. Amorphous insulin, assaying 19 units per mg., was also kindly supplied by Eli Lilly and Company.

RESULTS AND DISCUSSION

Upon incubation of insulin and insulin-I^{131} with liver extract, there was a rapid rise in the radioactivity in the TCA supernatant fluid. This is demonstrated in Fig. 1. The increased supernatant radioactivity was thought to be the result of proteolysis or direct deiodination of insulin-I^{131}. The latter mechanism seemed less likely because of the following observations. Based on the work of Harington and Neuberger (10), iodine substitution was probably exclusively on the 3 and 5 positions of the tyro-

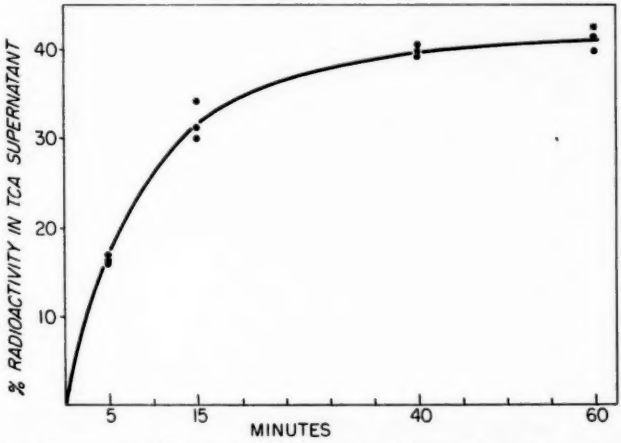

FIG. 1. Changes with time in per cent radioactivity in TCA supernatant fluid. 125 γ of insulin and 5 γ of insulin-I^{131} in 1 ml. of phosphate buffer, pH 7.5, were incubated with 1 ml. of extract. The extract was obtained after homogenizing rat liver with 3 times its weight of phosphate buffer, pH 7.5, then centrifuging. Values for triplicate samples were plotted to demonstrate reproducibility.

sine residues in this lightly iodinated insulin. Tong, Taurog, and Chaikoff (11) have reported that, although diiodotyrosine was readily deiodinated by rat liver slices, *N*-acetyldiiodotyrosine was not. This observation suggests that the free amino group is necessary in the deiodination of iodotyrosine by rat liver *in vitro*. Since insulin contains no *N*-terminal tyrosine residues, the possibility of a large degree of direct deiodination of insulin-I^{131} in rat liver extract appears unlikely.

An experiment concerning the question of direct deiodination of I^{131}-labeled proteins was performed with human serum albumin-I^{131} and ribonuclease-I^{131}. Unlike the result with insulin-I^{131}, there was essentially no radioactivity in the TCA supernatant solution after incubation of either substance with rat liver extract. This is shown in Fig. 2. This study

provided good evidence for the lack of an enzyme in liver extract capable of deiodinating I^{131}-labeled proteins and, therefore, supported the view that with insulin-I^{131} proteolysis was the main cause for the rise in radioactivity in the TCA supernatant solution. In addition, these results suggested the possibility that all native proteins might not be attacked by the rat liver system.

In order to determine whether non-labeled insulin was also a substrate for the enzyme system in question, the technique of competitive substrates (12) was utilized. Preliminary to this, it was essential to determine the

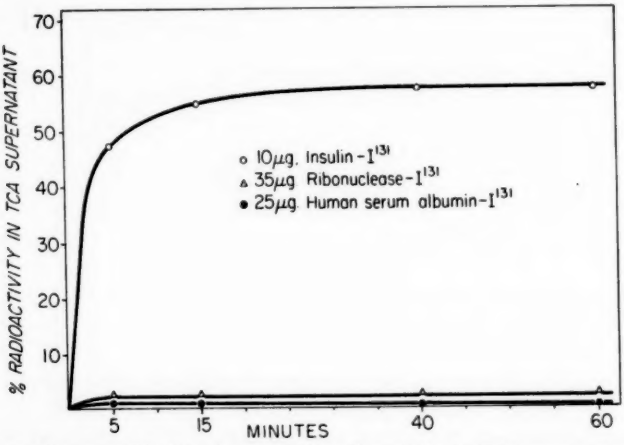

FIG. 2. Changes with time in per cent radioactivity in TCA supernatant fluid. Each iodinated protein in 1 ml. of phosphate buffer, pH 7.5, was incubated with 1 ml. of extract. Extract prepared as described in the legend of Fig. 1.

concentration of additional non-labeled insulin necessary in such a study. A plot of substrate concentration versus velocity is presented in Fig. 3. The ratio of insulin-I^{131} to amorphous insulin was the same at all levels of substrate concentration. Velocity was apparently proportional to the amount of substrate at lower concentrations, but this rate became less dependent on substrate concentration when, with increased substrate levels, the enzyme was apparently no longer present in excess. As a quantity of 125 γ of insulin resulted in a system in which the enzyme appeared not to be in excess, this amount of amorphous insulin was used in a competitive substrate study with a given amount of insulin-I^{131}. The addition of excess amorphous insulin, in addition to making the enzyme concentration limiting, increased the ratio of insulin to insulin-I^{131}. As shown in Fig. 4, this addition had a marked slowing effect on the rate of degradation

of a fixed amount of insulin-I^{131}. This effect was also noted when excess crystalline zinc insulin[1, 2] was used. These results indicated that non-labeled insulin was competing with insulin-I^{131} in its degradative system or was acting as an inhibitor to the system.

Experiments were performed in order to learn whether the above effect was the result of competition or inhibition. Amorphous insulin, in a

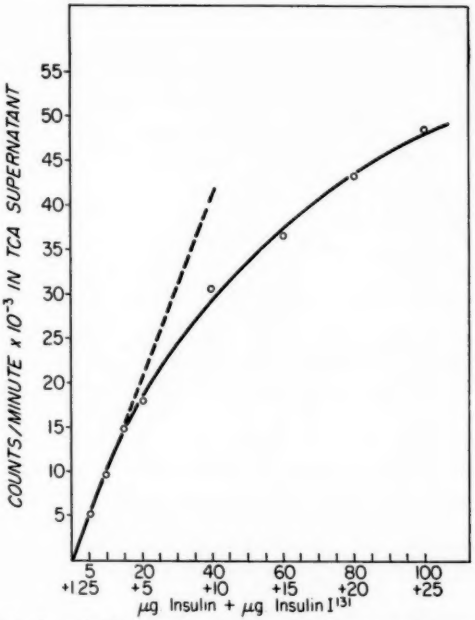

FIG. 3. Change in velocity with increasing substrate concentration. The substrates were dissolved in phosphate buffer, pH 7.5, and incubated for 5 minutes with 1 ml. portions of extract. Extract prepared as described in the legend of Fig. 1.

larger quantity, was incubated with a more concentrated liver extract and increase in non-protein nitrogen was determined by a semimicro-Kjeldahl determination of the TCA supernatant solution. As shown in Table I, there was a significant rise in the non-protein N over the control upon incubation of insulin with extract. Similar results were obtained when zinc insulin[1, 2] was used. In a study with amorphous insulin and insulin-I^{131}, increase in supernatant radioactivity and rise in non-protein N over control were determined with the same samples. As shown in Fig. 5, both

[2] Before use, crystalline zinc insulin was freed of zinc by dialysis in order to increase its solubility in phosphate buffer.

leveled off at approximately the same time, suggesting that each is a measure of insulin degradation.

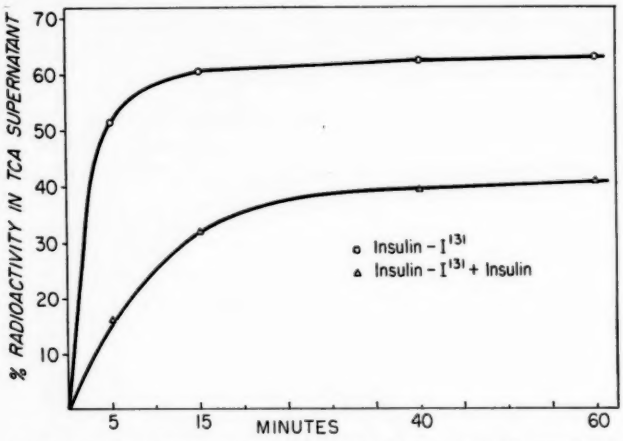

FIG. 4. Change with time in per cent radioactivity in TCA supernatant fluid. Incubation with 1 ml. of extract of 5 γ of insulin-I^{131} without and with 125 γ of insulin. Extract prepared as described in the legend of Fig. 1.

TABLE I

Rise in Non-Protein N upon Incubation of Insulin with Extract

The extract was obtained after homogenizing rat liver with an equal weight of phosphate buffer, pH 7.5, then centrifuging. 1 ml. of extract was incubated with 1 ml. of phosphate buffer, pH 7.5, with and without 3 mg. of insulin. Duplicate samples were run. The combined supernatant fluid of each sample was filtered before analysis.

System	Non-protein N		Increase in non-protein N
	0 min. incubation	30 min. incubation	
	γ	γ	γ
Without insulin	725	819	94
With insulin	732	968	236

The results of these experiments suggest that non-labeled insulin is proteolytically degraded by rat liver extract in competition with insulin-I^{131}.

Also, the radioactive insulin assay was compared directly with the rabbit hypoglycemia test. For the bioassay, a volume of incubation mixture was injected by cardiac puncture into fasted rabbits, each weighing approxi-

mately 2 kilos, such that a dose of preincubation potency of 2 units of insulin was given per kilo of body weight. Blood samples were drawn at 30 and 60 minutes after injection. The 30 and 60 minute blood sugar levels were expressed as a percentage of the preinjection level. The sum of these percentages has been designated by Mirsky and Broh-Kahn as "pooled percentage of the initial blood sugar" (8). Since this value has an inverse relationship with the biological potency of insulin, the pooled percentage approaches the uppermost value of 200 as the inactivation of insulin proceeds.

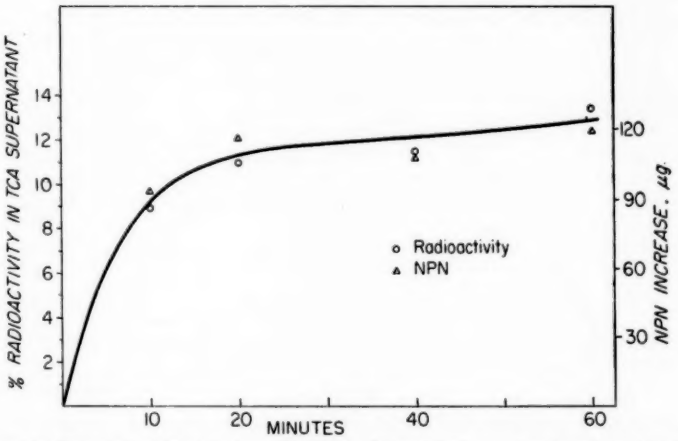

FIG. 5. Changes with time in non-protein N and per cent radioactivity in TCA supernatant fluid. 3 mg. of insulin and 10 γ of insulin-I^{131} in 1 ml. of phosphate buffer, pH 7.5, incubated with 1 ml. of extract. The extract was obtained after homogenizing rat liver with an equal weight of phosphate buffer, pH 7.5, then centrifuging.

At the time of injection, the remainder of each incubation mixture was added to TCA and assayed for percentage of radioactivity in the supernatant fluid.

The results of an experiment in which these two assays were carried out are presented in Fig. 6. These two methods of following the course of reaction yielded similar results.

This experiment in no way indicates the extent of degradation of insulin necessary for its complete biological inactivation. One reason is that a small scale insulin bioassay is a measure of its relative rather than its absolute amounts. Another consideration is the fact that the percentage of radioactivity in the TCA supernatant fluid does not reveal the exact amount of insulin degraded. The reason for this is that the location of

the iodinated tyrosyl residue or residues, the point or points of cleavage, and the solubility of the split-products in TCA have not been established. Nevertheless, these results do suggest that increased degradation as assayed by the rise in TCA-soluble radioactivity is associated with decreased biological activity.

As further comparison between these two assay methods, studies of various inhibitors with known effects on the insulin-inactivating action of liver extract were repeated. In these cases the assay for radioactivity was

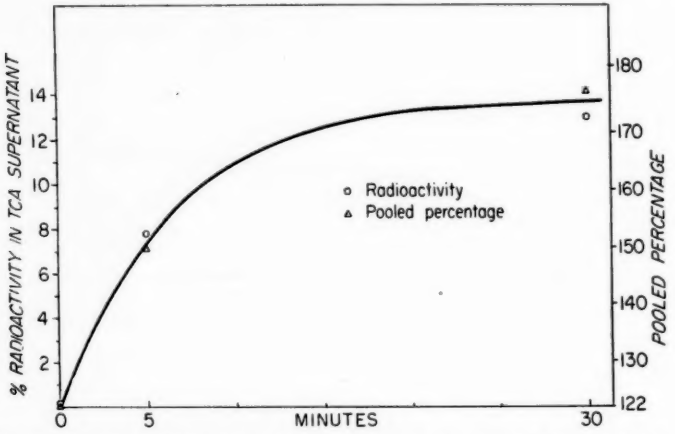

FIG. 6. Change with time in pooled percentage and per cent radioactivity in TCA supernatant fluid. 10 units of amorphous insulin and 5 γ of insulin-I^{131} in 1 ml. of water were incubated with 1 ml. of extract. The extract was obtained after homogenizing rat liver with 3 times its weight of water, then centrifuging. Extract adjusted to pH 7.5 with 1 N NaOH before use. Each point is the average value of five determinations. Fresh extract was prepared for each of the five sets of 5 and 30 minute incubations.

carried out and the results were compared with those of Mirsky and Broh-Kahn (8) in which the rabbit assay was used. The data are reported in Table II. Again, the similarity of the results of the two assay methods was demonstrated. Finally, since De Barbieri and Grassi (9) investigated proteolytic activity in rat liver extract at pH 3.5, the proteolysis of insulin-I^{131} at this pH was compared with that at pH 7.5. The latter is the pH which Shmidt and Saatchian (7) reported as optimal for insulin inactivation. The results in Table III indicate that insulin is probably not degraded at pH 3.5.

Proteolysis of insulin by rat liver extract at pH 7.5 may involve cleavage of peptide bonds alone or, in addition, may include the splitting of disulfide

bonds. Some studies on the substrate specificity of this insulin-inactivating system have been described in the preliminary communication (1). It appears that several native proteins are not susceptible to rapid attack by the enzyme or enzymes in question. Further work will be carried out

TABLE II

Comparison of Radioactive and Biological Methods for Detecting Inhibitory Effect of Various Substances on Insulin-Inactivating System

The extract was obtained after homogenizing rat liver with 3 times its weight of water, then centrifuging. The extract was adjusted to pH 7.5 with 1 N NaOH before use. 8 units of insulin, 5 γ of insulin-I^{131}, and test substance in 1 ml. of aqueous solution were incubated with 1 ml. of extract for 30 minutes. The pooled percentage values were those obtained by Mirsky and Broh-Kahn (8).

Substance	Final concentration	Pooled percentage	Per cent radioactivity in TCA supernatant
None		195	22
Manganous sulfate	0.001 M	200 *Ca.*	21
Soy bean inhibitor	0.45 mg.	212	21
Cupric sulfate	0.0005 M	91	9
Zinc sulfate	0.0005 "	118	5
Iodoacetate	0.001 M	117	20*
Iodosobenzoate	0.001 "	84	13

* Iodoacetate in the concentration of 0.001 M was slightly, though consistently, inhibitory. At 0.002 M, the inhibition was marked (13 per cent radioactivity in the TCA supernatant fluid).

TABLE III

Comparison of Degradation of Insulin-I^{131} at pH 3.5 and 7.5

The extract was obtained after homogenizing rat liver with 1.5 times its weight of water. 0.5 ml. of extract was incubated with 10 γ of insulin-I^{131} in 1.5 ml. of solution of buffer. Period of incubation, 30 minutes.

pH	Buffer	Per cent radioactivity in TCA supernatant
3.5	0.2 M phthalate	<1
7.5	0.067 M phosphate	29

in order to determine the nature of the labile linkage or linkages in insulin. For this purpose, a program concerned with the purification of the enzyme or enzymes involved has been initiated.

SUMMARY

Evidence is presented indicating that insulin is probably proteolytically inactivated by rat liver extract at pH 7.5.

The authors are indebted to Dr. N. J. Elgee and Miss Ruthe Bailey for performing the insulin bioassays.

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THE EFFECT OF AZIDE ON PHOSPHORYLATION ACCOMPANYING ELECTRON TRANSPORT AND GLYCOLYSIS*

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The dissociation of respiration from synthetic processes by azide was shown by studies of Clifton in 1937 (1) and by the results of subsequent investigations (2–4). Hotchkiss in 1944 made the significant observation that, although bacterial respiration was enhanced by azide, inorganic orthophosphate (P_i) uptake was decreased (5). The ability of azide to uncouple oxidation of substrates from net adenosinetriphosphate (ATP) synthesis in intact cells and cell-free preparations has been well established; present evidence suggests that the uncoupling action of azide, like that of 2,4-dinitrophenol, is on the electron transport between reduced coenzymes and oxygen (6–9). The uncoupling would result if a phosphorylated intermediate were rendered unstable or if the initial uptake of P_i did not occur although electron transfer continued. Experiments labeled with P_i labeled with P^{32} and O^{18} which favor the latter possibility are presented in this paper.

A second objective was to examine the relation of azide to phosphorylations accompanying anaerobic glycolysis. Data obtained by Spiegelman, Kamen, and Sussman have been interpreted as showing that azide dissociates anaerobic glycolysis from ATP synthesis (3). This suggestion, which has been accepted in more recent publications (10, 11), is considered untenable in view of the experimental results presented herein.

Materials and Methods

Manometric measurements were made with the Warburg apparatus by conventional techniques. P_i was determined essentially as described by Lowry and Lopez (12) or with the use of ferrous sulfate as a reducing agent according to Sumner (13). In experiments with yeast acetone powders chilling of the flasks before addition of an equal volume of 0.6 M trichloroacetic acid was necessary to obtain clear supernatant solutions. The

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enzymic reaction was conveniently stopped by addition of sufficient HCl to lower the pH to about 3 before chilling the flasks.

Sodium azide (Eastman) was used without further purification in the experiments with yeast and after crystallization from ethanol-water in experiments with liver mitochondria.

Mitochondria were prepared from fasted rat livers which were minced and ground in 0.25 M sucrose containing 10^{-4} M ethylenediaminetetraacetate essentially as described by Schneider (14), except that the initial residue of nuclei and cellular débris was not reextracted. The fluffy layer of microsomes was swirled off and discarded after sedimentation of the mitochondria in a Servall high speed centrifuge and the mitochondria were washed twice by resuspension in the sucrose medium. For the enzyme runs the washed mitochondria were resuspended in a volume (in ml.) of 0.25 M sucrose equal to the gm. of fresh liver. Emphasis in the preparation was on isolation of "clean" mitochondria, not on isolation of high yields.

The yeast acetone powder was prepared by a modification of the procedure of Hochster and Quastel (15). A suspension of fresh brewers' yeast was chilled to 6° and filtered through a Büchner funnel in a cold room, and the yeast was washed three times by suspension in ice-cold 0.85 per cent NaCl, stirred for 5 minutes, and refiltered. 20 gm. portions of the washed yeast were ground vigorously for 10 minutes in a chilled Pyrex mortar with an approximately equal volume of 20 to 30 mesh Pyrex glass and 10 ml. of 0.11 M nicotinamide solution, pH 7.0. The ground yeast was mixed with an equal volume of 0.85 per cent NaCl and the slurry was centrifuged. The milky supernatant liquid was poured into 6 volumes of vigorously stirred ice-cold acetone. The precipitate was collected by centrifugation, washed twice by suspension and centrifugation from cold acetone, transferred to a Büchner funnel with aid of acetone, and washed with cold acetone. The acetone was removed by drawing air through the cake, and the product was dried in a vacuum desiccator over P_2O_5 and stored in a refrigerator. Before use the acetone powder was triturated in a ratio of 25 mg. of powder per 0.3 ml. of water.

For measurement of the amount of P^{32} present in ATP, the ATP in the trichloroacetic acid supernatant solutions was adsorbed on 100 mg. of charcoal (16), followed by three careful washings to remove P_i. The washed charcoal was transferred to a planchet with the aid of several small volumes of alcohol, and the samples were dried under an infra-red lamp and counted with a Geiger tube by conventional techniques. Azide did not interfere with the adsorption of ATP on the charcoal. Measurements of the amount of O^{18} in the inorganic phosphate were made by procedures which allowed use of samples of 50 to 100 μmoles of phosphate without further dilution (17); the estimated maximal error of the results given in Table II is about 5 per cent.

Results

Azide and Inorganic Phosphate Requirement of Glycolysis—The suggestion that azide may cause formation of P_i from 1,3-diphosphoglycerate in the presence of the enzyme 3-phosphoglycerate kinase (3), or formation of P_i accompanying any of the reactions of glycolysis, may be conveniently checked by measurement of the dependence of glycolysis upon P_i in the presence of high concentrations of azide. In yeast extracts deficient in phosphatase activity, which metabolize glucose in accordance with the well known Harden-Young equation, 2 glucose + 2 phosphate $\rightarrow$ $2CO_2$ + hexose diphosphate + $2C_2H_5OH$ + $2H_2O$, the fermentation rate decreases as the supply of phosphate is exhausted, even though excess glucose remains (18, 19). Any agent that would dissociate glycolysis from P_i uptake or hasten the liberation of P_i from organic combination would permit prolonged and enhanced CO_2 production from systems deficient in P_i or in phosphate acceptors.

The results given in Fig. 1 clearly demonstrate the inability of azide to reduce the P_i requirement for glucose fermentation by a suitable yeast extract preparation. Even in the presence of 0.025 M azide the rate of CO_2 production fell off as the P_i supply was exhausted and was stimulated by addition of excess P_i in a manner entirely comparable to that found in the absence of azide. The results reported in Fig. 1 were obtained with air as a gas phase; entirely similar results were obtained with 95 per cent N_2-5 per cent CO_2 as the gas phase.

Azide and Phosphate Acceptor Requirement for Glycolysis—A further test of the possibility that azide may accelerate the liberation of P_i from phosphorylated intermediates and the possibility that azide might act as a phosphate acceptor in reactions accompanying glycolysis was made with a reaction system similar to that used for the experiments reported in Fig. 1, except that fructose-1,6-diphosphate was used as a substrate in the presence of excess P_i. Under these conditions the rate of CO_2 production is limited by the action of phosphatases to give phosphate acceptors (19). The data presented in Fig. 2 show that the low rate of CO_2 production was actually slightly decreased by the presence of azide. The dependence of the rate of CO_2 production upon a phosphate acceptor is shown by the marked acceleration of the rate following addition of glucose. Excess fructose-1,6-diphosphate was already present; thus the contribution of glucose was other than as a source of metabolites for CO_2 production.

Effect of Azide on Enzymic Hydrolysis of ATP and Other Phosphates—The rate of hydrolysis of ATP by washed liver mitochondria is greatly accelerated by 2,4-dinitrophenol (8, 20–22). Preliminary experiments showed that azide over a range of concentrations did not cause a hydrolysis of ATP comparable to that produced by 2,4-dinitrophenol but that 3 to

6×10^{-4} M azide did increase ATP hydrolysis by liver mitochondria. This suggested that the higher concentrations of azide might be inhibitory to the reactions causing formation of P_i from ATP; subsequent experiments showed that 5×10^{-3} M azide blocked the action of 2,4-dinitrophenol. Representative results showing the effects of azide are given in

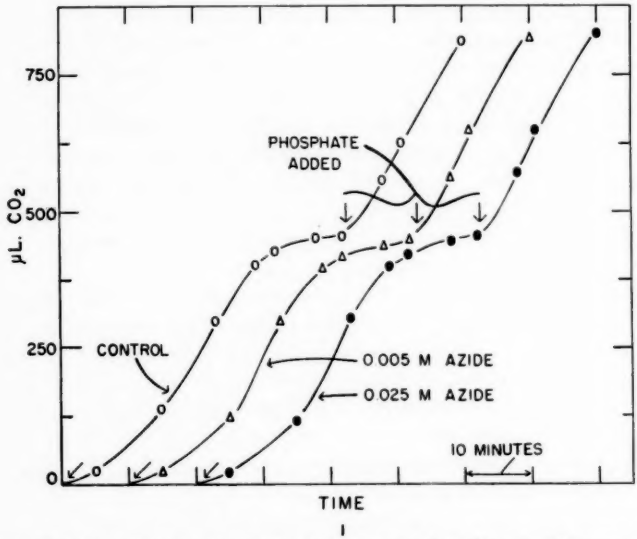

FIG. 1. Inability of azide to reduce the inorganic phosphate requirement for glucose fermentation by yeast extract. To the main compartment of each Warburg flask were added 200 μmoles of glucose, 1 μmole of ATP, 0.8 μmole of diphosphopyridine nucleotide, 1 μmole of $MgSO_4$, 10 μmoles of potassium phosphate, 25 mg. of an acetone powder of yeast extract, and azide as indicated in a total volume of 1.0 ml. at pH 6.5. Following addition of the acetone powder the flasks were incubated for 15 minutes at 30°, and the stop-cocks were closed as indicated by the lowest arrows. At the times indicated, 16.5 μmoles of potassium phosphate, pH 6.5, in 0.2 ml. were added from the side arm. The gas phase was air.

Table I. These data show the marked acceleration of ATP hydrolysis by 3×10^{-5} M 2,4-dinitrophenol, the smaller acceleration by 5×10^{-4} M azide, and the small or negligible effect of 5×10^{-3} M azide. In addition the data show that 5×10^{-4} M azide partially blocked and 5×10^{-3} M azide nearly completely blocked the increase in ATP hydrolysis in the presence of dinitrophenol.

Early in the studies of the action of azide, the possibility that azide might accelerate the action of phosphatases was investigated by measurement of the rate of hydrolysis of various organic phosphates by intestinal

phosphatase. The experiments showed no acceleration of the hydrolysis rate and will not be reported in detail here. Under the conditions used, 0.05 M azide at pH 9.3 had no effect on the rate of hydrolysis of 0.0012 M ATP by intestinal phosphatase and inhibited the hydrolysis of 0.00125

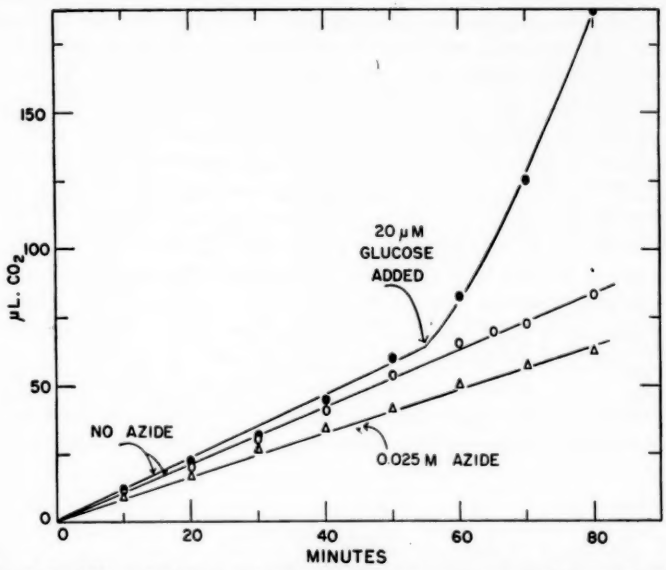

Fig. 2. Inability of azide to reduce the phosphate acceptor requirement for hexose diphosphate fermentation by yeast extract. To the main compartment of each Warburg flask were added 50 μmoles of fructose-1,6-diphosphate, 1 μmole of ATP, 1 μmole of $MgSO_4$, 0.5 μmole of diphosphopyridine nucleotide, 10 μmoles of potassium phosphate, and azide as indicated in a total volume of 1.7 ml. at pH 6.5, except for one flask which had a total volume of 1.5 ml. in the main compartment and 0.2 ml. of glucose solution in the center well. Following 15 minutes equilibration at 30°, 0.3 ml. of a suspension containing 25 mg. of an acetone powder of yeast extract was added from the side arm and measurement of CO_2 output commenced 5 minutes later; 55 minutes later glucose was added to one flask from the center well. The gas phase was 95 per cent N_2-5 per cent CO_2.

M fructose-1,6-diphosphate by 21 per cent and the hydrolysis of 0.012 M β-glycerophosphate by 42 per cent.

Inhibition of P^{32} and O^{18} Exchange Reactions by Azide—Experiments reported elsewhere have demonstrated that washed liver mitochondria, in the absence of oxygen uptake, catalyze a rapid exchange of P_i with ATP and of the oxygens of P_i with water (23). The oxygen exchange reaction has been attributed principally to the reversibility of the primary

TABLE I

Effect of Azide and 2,4-Dinitrophenol on Phosphate Cleavage from ATP by Liver Mitochondria

To 0.6 ml. of a solution at pH 7.4 containing 6.5 μmoles of potassium ATP, 7.5 μmoles of $MgSO_4$, 75 μmoles of KCl, and 1 μmole of potassium phosphate were added 0.2 ml. of washed liver mitochondria in 0.25 M sucrose, 10^{-4} M ethylenediaminetetraacetate, and 0.005 M β-glycerophosphate, pH 7.2. The mixture was incubated at 28° for 7 minutes, then water, azide, or dinitrophenol solution was added as indicated to give a final volume of 1.0 ml. After an additional 15 minute incubation, 1.0 ml. of cold 10 per cent trichloroacetic acid was added, and P_i was determined on an aliquot of the supernatant liquid. The values for P_i are averages of duplicate analyses; variation of duplicates was less than 2 per cent.

Min. after adding azide or dinitrophenol	Azide	2,4-Dinitrophenol	P_i present	P_i formation caused by azide or dinitrophenol
	M	*M*	*μmoles*	*μmoles*
0	0	0	1.48	
15	0	0	2.18	
15	5×10^{-4}	0	2.80	0.62
15	5×10^{-3}	0	2.26	0.08
15	0	3×10^{-5}	6.06	3.88
15	5×10^{-4}	3×10^{-5}	4.24	2.06
15	5×10^{-3}	3×10^{-5}	2.20	0.02

TABLE II

Effect of Azide on P^{32} and O^{18} Exchange Reactions Catalyzed by Mitochondria

The reaction tubes contained in 3 ml. of total volume, pH 7.4, 19 μmoles of potassium ATP, 22 μmoles of $MgSO_4$, 150 μmoles of KCl, 0.6 ml. of washed liver mitochondria in 0.25 M sucrose containing 10^{-4} M ethylenediaminetetraacetate, and 45 μmoles of potassium phosphate labeled with O^{18} and P^{32}. The potassium phosphate was added after a preliminary incubation period of 7 minutes, the samples were incubated for an additional 15 minutes at 25°, and then 1 ml. of 30 per cent trichloroacetic acid was added. P^{32} present in the ATP and O^{18} in inorganic phosphate were determined on appropriate aliquots.

Min. after adding P_i^{32}-O^{18}	Azide	P_i present	Radioactivity in ATP	Atom per cent excess O^{18} in inorganic phosphate
	M	*μmoles*	*c.p.m.*	
0	0	45.0	32	0.35
15	0	47.4	1717	0.22
15	5×10^{-6}	47.0	1670	
15	5×10^{-5}	47.0	1568	
15	5×10^{-4}	48.2	767	
15	5×10^{-3}	45.5	242	0.33

reaction or reactions by which energy derived from electron transport is coupled to uptake of P_i. Interference of azide in oxidative phosphorylation would be expected to decrease the P_i^{32}-ATP exchange reaction. If azide blocked the initial reaction (or reactions) of P_i uptake, then the phosphate oxygen exchange should likewise be inhibited.

Two preliminary experiments showed that 0.005 M azide inhibited markedly both the exchange of P_i^{32} with ATP and the exchange of phosphate oxygens catalyzed by liver mitochondria. The experiments reported in Table II show the effect of graded levels of azide on the exchange reaction of P_i^{32} with the phosphate groups of ATP, and the effect of 0.005 M azide on the exchange of phosphate oxygens in the absence and in the presence of azide. For these experiments P_i labeled with P^{32} and O^{18} was incubated with ATP and washed liver mitochondria under the conditions given in Table II. The results show an increasing inhibition of the P_i-ATP exchange with increase in azide concentration. The sample incubated in the absence of azide showed the expected marked exchange of P_i-oxygen; the decrease in O^{18} content was equivalent to an exchange of 67 microatoms of phosphate oxygen. The P_i oxygen exchange, as well as the P_i-ATP exchange, was nearly completely inhibited by 5×10^{-3} M azide. It is of interest that the P_i^{32}-ATP exchange reaction is still considerable in the presence of a concentration of azide (5×10^{-4} M) which caused acceleration of ATP hydrolysis.

DISCUSSION

Interpretation of the effects of azide on the P_i^{32}-ATP and the P_i-O^{18} exchanges requires an understanding of the reactions involved. These exchange reactions occur without net oxygen uptake and in the absence of added substrates. Evidence, but not conclusive proof, has been obtained that the exchanges result from a reversal of the reactions by which P_i uptake is coupled to electron transport, the O^{18} exchange resulting largely from reversal of the reaction or reactions by which P_i is incorporated into an intermediate and the P^{32} exchange from reversal of the over-all reactions by which P_i is incorporated into ATP (23). If these premises are correct, interference of azide with the P_i^{32}-ATP exchange reaction would be an expected result in view of the known uncoupling of oxidative phosphorylation by azide (6–8). Measurement of the P_i^{32}-ATP exchange would thus give an evaluation of the effect of azide on oxidative phosphorylation independent of its known inhibition of cytochrome oxidase. Further, if the proposed mechanism of the O^{18} exchange is valid, the ability of azide to inhibit this O^{18} exchange means that azide can prevent the initial uptake of P_i coupled with electron transport. This would occur if, in the presence of azide, electron transport continued without participation of

P_i in the reaction. A similar suggestion has been made for the action of dinitrophenol (23). The need for P_i when glutamate is oxidized by kidney homogenates in the presence of azide (6) may result from the requirement of P_i for the phosphorylation accompanying α-ketoglutarate oxidation at the substrate level; this interpretation is supported by the observations of Judah (7). The hypothesis that the uncoupling agents prevent the initial uptake of P_i is in contrast to the possibility that they cause release of P_i from a phosphate-containing intermediate.[1]

The demonstration that azide at appropriate concentrations can accelerate the cleavage of P_i from ATP by liver mitochondria is of importance in the understanding of the biological effects of azide. The depletion of cellular ATP by azide offers a logical explanation for the observations of various workers showing an increased rate of fermentation and utilization of carbohydrate reserves in the presence of azide (2, 10, 24, 25). Increased substrate utilization in the presence of appropriate concentrations of azide would be anticipated in any system in which the rate of substrate utilization was dependent upon the availability of phosphate acceptors or P_i.

The acceleration of the rate of formation of P_i from ATP by azide follows logically from the premise that under appropriate conditions P_i and ATP are in dynamic equilibrium through reversal of the reactions by which ATP formation is coupled to electron transport. For such a dynamic equilibrium to exist, energy derived from the formation of P_i must be preserved in some manner (for example, by reduction of an electron carrier or formation of an energy-rich intermediate not containing the P_i) in order for the P_i to be reincorporated into the ATP. In the presence of azide, P_i formed from ATP would not be taken up again because the azide allows the oxidation to occur without P_i uptake or in some other manner causes dissipation of the energy derived from the ATP cleavage. Similar considerations apply to the action of 2,4-dinitrophenol. The net breakdown of ATP would thus not result primarily from an increased rate of formation of P_i but from prevention of the reincorporation of the P_i formed into ATP.

On the basis of present evidence, the hypothesis that azide in intact cells interferes with phosphorylation accompanying glycolysis is unwarranted. The results given in this paper showing the strict dependence of anaerobic glycolysis by yeast extract on adequate concentrations of inorganic phosphate and phosphate acceptors in the presence of high azide concentrations demonstrate conclusively that azide does not interfere with phosphate esterification or transfer reactions by such extracts. Spiegel-

[1] Recent data have shown that the P_i-ATP exchange is more sensitive to the inhibitory effects of 2,4-dinitrophenol and of azide than is the P_i oxygen exchange. Details of these experiments and their significance will be presented later.

man *et al.* mention that 5×10^{-3} M azide did not inhibit phosphate esterification with a yeast extract preparation (3). None the less, these authors suggested that in intact yeast "azide uncouples anaerobic oxidation from synthesis by means of a replacement reaction between azide and the acyl phosphate of diphosphoglycerate while the latter is combined with phosphoferase." They regarded it likely that excessive amounts of dissolved protein in their cell-free preparations bound azide and thus prevented its inhibitory effect. Binding of azide by proteins is highly untenable as an explanation for the lack of effect of the high concentrations of azide used in the experiments reported herein. If the yeast acetone powder used were all protein with an average molecular weight of 100,000, each protein molecule would need to inactivate nearly 100 azide molecules in the experiment reported in Fig. 2 in which 0.025 M azide was used. Further, the ability of much lower azide concentrations, *e.g.* 5×10^{-4} M, to dissociate respiration from phosphorylation in intact cells and in liver mitochondria shows that low concentrations of azide may be effective in the presence of considerable protein. Postulation of a greater sensitivity to azide of glycolytic enzymes in the intact cell than in cell-free extracts is without experimental justification.

Observations cited in support of the contention that azide dissociates anaerobic glycolysis from phosphate uptake (3) are all indirect and may have their explanation in other effects of azide. The depletion of the ATP reservoirs of the intact yeast in the presence of azide would offer a possible explanation for many of the observed effects. In many respects the effect of azide on intact cells is similar to that of 2,4-dinitrophenol; unpublished results from this laboratory and data of other workers (7, 22) have demonstrated that phosphate uptake and transfer reactions associated with glycolysis are not affected by 2,4-dinitrophenol.

The actions of azide and of 2,4-dinitrophenol on oxidative phosphorylation are, however, not entirely analogous. The blocking by azide of the acceleration of P_i formation from ATP in the presence of 2,4-dinitrophenol (Table I) shows clearly that azide has an inhibitory effect not shared by 2,4-dinitrophenol. The rate of ATP breakdown is not decreased by increase in the 2,4-dinitrophenol concentration above that required for a nearly maximal effect (20). The secondary effect of azide is probably responsible for the observation that acceleration of the rate of P_i formation from ATP, although appreciable at 5×10^{-4} M azide, was small at 5×10^{-3} M. Azide will block oxygen uptake (26) and its properties responsible for this blocking may be related to the inhibitory effect on ATP breakdown. In addition, Meyerhof has demonstrated inhibition of an "ATPase" of yeast by azide (19), and, as shown herein, azide will inhibit intestinal phosphatase. Thus, although azide and 2,4-dinitrophenol both have the

ability to uncouple phosphorylation from electron transport, their effects on intact cells may differ; such differences have been reported (4, 27).

The technical assistance of Mrs. Madelyn Ferrigan and Mr. A. Lajeune is gratefully acknowledged.

SUMMARY

Azide at concentrations as high as 0.025 M is without effect on the requirement for phosphate acceptors or inorganic phosphate for glycolysis by a yeast extract. From these results and other considerations the conclusion is reached that present evidence does not warrant postulates that azide interferes with phosphorylations associated with glycolysis in extracts or intact cells.

The reversible exchange of P^{32} of inorganic phosphate with ATP catalyzed by washed liver mitochondria, as well as the more rapid exchange of phosphate oxygen as measured with O^{18}, was inhibited by 5×10^{-3} M azide. If, as has been suggested, these exchange reactions result primarily from a dynamic reversal of oxidative phosphorylation, then azide prevented the initial uptake of inorganic phosphate coupled with electron transport.

Azide caused an appreciable increase in the rate of net formation of inorganic phosphate from ATP by liver mitochondria which was maximal at about 5×10^{-4} M azide but small at 5×10^{-3} M azide. The much more marked acceleration of inorganic phosphate accumulation in the presence of 3×10^{-5} M 2,4-dinitrophenol was partially blocked by 5×10^{-4} M azide and nearly completely blocked by 5×10^{-3} M azide. The accumulation of inorganic phosphate may result from a reversal of the phosphate incorporation into the ATP coupled with electron transport and prevention of the uptake of the inorganic phosphate formed. Azide is regarded as having a secondary inhibitory effect, not shown by 2,4-dinitrophenol, on the reactions involved.

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THE HORMONAL CONTROL OF TRYPTOPHAN PEROXIDASE IN THE RAT*

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Adrenalectomized rats developed an increased activity of the liver tryptophan peroxidase-oxidase system (TPO) 5 to 7 hours after the intraperitoneal injection of tryptophan, but not after the administration of compounds which were not substrates of the enzyme (1). This change in TPO activity was analogous to the substrate-induced enzyme adaptations frequently encountered in microorganisms (2). In intact rats the TPO activity was increased by the administration of other compounds such as histidine, epinephrine, and histamine, as well as tryptophan. These non-substrate compounds appeared to act in a second way which was distinguished from the substrate-induced adaptation by the production of generally smaller increases in TPO activity and by their action only in the presence of adrenal glands.

It was considered that all of the effective non-substrate compounds could cause directly or indirectly an increased secretion by the adrenal cortex. The present study of the effect of histidine identified its mode of increasing the TPO activity as a hormone-induced adaptation, which could be duplicated by the administration of cortisone. Recent studies in this and other laboratories (3–6) have also indicated that the glucosteroids and certain other hormones can influence the TPO activity of the liver.

EXPERIMENTAL

Male and female Wistar rats weighing between 140 and 150 gm. were used. The tryptophan peroxidase level was assayed in duplicate in the individual livers of the rats in all groups. The test compounds were administered by intraperitoneal injection. The treated animals were killed for assay 5 hours after the last injection, when near maximal increases in the tryptophan peroxidase level were expected (7). The adrenalectomies were performed under ether anesthesia, usually 4 days before the rats were

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used. The animals were given 1 per cent NaCl as drinking water unless they received cortisone.

Tryptophan Peroxidase Assay—The activity of the tryptophan peroxidase was measured by the spectrophotometric determination of the amount of kynurenine formed during the aerobic incubation of liver homogenate with L-tryptophan. The method was similar to the methods used earlier (1, 3, 7); these have not previously been described in detail.

Preparation of Homogenate—To avoid any loss of activity, the assay of the liver homogenates was started within 30 minutes after the death of the rats. The livers were promptly removed from the decapitated animals, chilled in ice water, and weighed to 0.1 gm. They were homogenized in a Waring blendor for 2 minutes at 2° with 7 volumes of cold 0.14 M KCl containing 0.0025 N NaOH. The homogenate was allowed to stand briefly at 2° before use.

Reactions—The homogenate from each animal was tested in two concentrations, with corresponding blanks containing no tryptophan. The 20 ml. reaction beakers contained 1 ml. of 0.2 M phosphate buffer, pH 7.0, 1 or 2 ml. of homogenate, 0.3 ml. of 0.03 M L-tryptophan (replaced by water in the blanks), and water to a total volume of 4.0 ml. The homogenate was added last, after preliminary temperature equilibration of the other components. The reactions were run in a Dubnoff shaker at 38° in an atmosphere of O_2. 1 hour after addition of the homogenate, the reaction was stopped with 2.0 ml. of 15 per cent metaphosphoric acid. The reaction mixtures then were filtered.

The dry weight, less the added KCl, was determined from duplicate 2 ml. samples of each homogenate dried for 2 or 4 hours at 110°.

Determination of Kynurenine—3 ml. of each reaction filtrate were adjusted to pH 6.5 to 7.5 by the addition of 1.0 ml. of NaOH of an appropriate concentration, about 1.1 N, with the usual sample of metaphosphoric acid. The neutral solution was placed in a 1 cm. cell and read against water at 365 mμ in a Beckman spectrophotometer. The amount of kynurenine formed was calculated from the optical density of the experimental filtrate minus its blank, by the use of the molar extinction coefficient of kynurenine under these conditions (ϵ = 4540 (1 mole per liter, 1 cm. depth)). The activity of the homogenate was expressed as the number of μmoles of kynurenine formed per gm. of dry weight per hour. The values determined from the two concentrations of each homogenate were averaged.

Validity of Method—Several types of evidence showed that the amount of kynurenine accumulated under these conditions and measured as described above adequately indicated the amount of tryptophan oxidized in the tryptophan peroxidase reaction. The immediate oxidation product,

formylkynurenine, did not accumulate because a large excess of kynurenine formamidase was always present in the crude liver preparation (7). The method of deproteinization with metaphosphoric acid gave superior recoveries of kynurenine, as compared with the zinc acetate-NaOH method used previously. The absence of any kynurenine losses during deproteinization was demonstrated by the complete recovery (100 ± 2 per cent) of a range of amounts between 0.2 and 2.3 μmoles of DL-kynurenine added to the reaction mixture. No products of tryptophan oxidation besides kynurenine could be found by the following methods: determination of anthranilic acid by ether extraction and diazotization (8); spectrophotometric determination of anthranilic and kynurenic acids (8); paper chromatography of the reaction filtrates by Dalgliesh's method (9).

As a final check of the completeness of kynurenine accumulation in the system, kynurenine was trapped as it was formed by the addition of an excess of purified kynureninase from *Pseudomonas fluorescens* (10). Anthranilic acid was then formed quantitatively, in the same amounts as the kynurenine which accumulated in the control reactions under the usual conditions of the assay.

The reliability of the kynurenine measurements and the use of optimal conditions for the enzyme reaction lent validity to the observed changes in enzyme activity. Since under the assay conditions the activity of the enzyme was proportional to its concentration, it seemed probable that the activity changes resulted from changes in the enzyme concentration. Final proof of this point will require determination of the enzyme by a method which does not involve activity measurements.

Results

The levels of tryptophan peroxidase activity found in the different groups of animals and the significance of the differences between the groups are summarized in Fig. 1. Both histidine and cortisone caused about the same degree of enzyme increase when either was administered to intact animals 5 hours before death. When both compounds were given together, there was no further increase over the level caused by histidine alone. Adrenalectomy abolished the effect of histidine, but not that of cortisone. A further indication of the rôle of the adrenal glands in the maintenance of the tryptophan peroxidase level was the significant lowering of the enzyme in the adrenalectomized as compared with the intact animals. A similar observation was made earlier (1).

In the intact animals there was no summation of the effects of cortisone and histidine, although in the adrenalectomized animals the effect of the two substances was greater than that of cortisone alone. In the intact animals the effect of histidine alone or of histidine plus cortisone was also

somewhat greater than that of cortisone alone. It would appear that a part of the effect of histidine on the enzyme level was not referable to cortisone, although it was manifested only in the presence of cortisone.

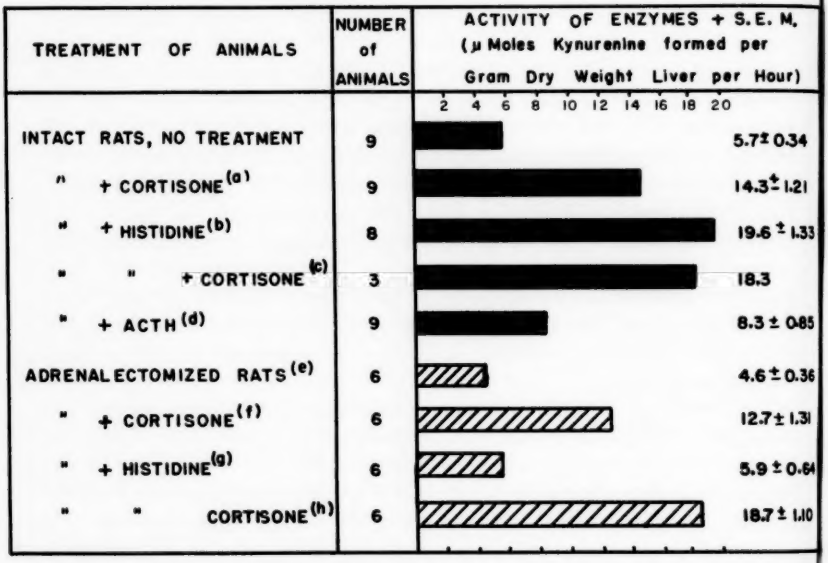

FIG. 1. Activity of tryptophan peroxidase in groups of rats. Adrenalectomized rats were assayed on the 4th post-operative day unless otherwise stated; all animals were assayed 5 hours after the last treatment. The differences in treatment within a group had no apparent effect on the enzyme activity; those between the untreated and treated groups of both the intact and the adrenalectomized animals were all significant (P = or < 0.01), except for the adrenalectomized rats given histidine ($P = 0.1$); also significant were differences between the untreated intact and adrenalectomized rats ($P = 0.02$), between the intact rats treated with cortisone and those treated with histidine ($P < 0.01$), and between the adrenalectomized rats treated with cortisone and those treated with cortisone plus histidine ($P < 0.01$). (*a*) Single daily intraperitoneal injection of 1.25 mg. of cortisone (Cortone acetate, Merck) in 0.5 ml. of saline for 1, 2, and 3 days each to one-third of the group; (*b*) single dose of 310 mg. of L-histidine·HCl·H_2O intraperitoneally in a final volume of 5 ml., adjusted to pH 7 with NaOH; (*c*) histidine on 3rd day of cortisone treatment (*a*); (*d*) 10 units per day for 5 days, a single injection of 10 units, and a single injection of 2 units of "National" ACTH solution intraperitoneally to subgroups of three rats; another group received a single subcutaneous injection of 2 units of ACTH (Acthar, Armour) in 16 per cent gelatin (not included); (*e*) 1 per cent NaCl as drinking water; (*f*) one rat treated as in (*a*) for 5 days, three rats for 2 days, and two rats for 4 days; (*g*) same as (*b*); (*h*) histidine (*b*) to four rats on 4th day, and to two rats on 5th day of cortisone treatment (*a*).

Corticotropin (ACTH), administered either in isotonic saline solution or as a suspension in gelatin, increased the enzyme level significantly but not to the same absolute level as did cortisone. Quantitative differences in the effects of these compounds have been noted before (11). Corticosterone, which is the usual glucosteroid elaborated by the adrenals of some rats (12), was also tested in three animals in the same concentration as was cortisone, and was found to be somewhat less active than cortisone. In the absence of time and dosage studies for both compounds, no comparison of their relative effectiveness can be made.

Other experiments showed that various stress stimulations of the hypophyseal-adrenal axis also increased the tryptophan peroxidase. For example, the injection of 5 ml. of solutions of alanine and of NaCl which were twice isotonic in concentration increased the enzyme levels to 12.2 and 9.8 μmoles per gm. per hour, respectively, in groups of three rats. Injection of isotonic NaCl solutions had no effect on the enzyme level.

The addition *in vitro* of cortisone or histidine did not alter the activity of the homogenates.

DISCUSSION

The major effect of histidine on the TPO level can apparently be attributed to the eventual release of adrenal hormones in the histidine-treated animals. Thus, both histidine and cortisone caused increases of a similar magnitude within the same time; their effects did not summate, and the effect of histidine was abolished in the adrenalectomized animals while that of cortisone persisted. The treatments which have resulted in increased TPO activity can therefore be separated into the administration of the substrate, causing substrate-induced adaptation, and a variety of treatments causing hormone-induced adaptation. The latter included the administration of the adrenal hormones, cortisone, hydrocortisone (6), and ACTH (4), and treatment with histidine or injections of hypertonic solutions, which caused the release of these hormones. Epinephrine and histamine administration (1) and x-irradiation or administration of a variety of stressing drugs (6) also have caused an increased TPO activity only in the presence of the adrenal glands. These agents presumably also acted by causing a release of adrenal cortical hormones. Stress, epinephrine, and histamine do cause ACTH release from the pituitary (13, 14). Part of the x-ray effect persisted after hypophysectomy (6), and this part may have resulted from a direct stimulation of the adrenals. Still other ways of altering the TPO level which do not involve either the substrate- or the adrenal hormone-induced mechanisms were suggested by that part of the histidine effect which was greater than the effect of cortisone, and by the action of growth hormone and other endocrines on the enzyme level (3, 5).

The hormone-induced adaptation of TPO studied here can at present be distinguished from the substrate-induced adaptation only by the way in which it is produced, although the two effects do differ in magnitude (1, 6). The increased activity in each instance appeared to be caused by an increase in the enzyme concentration. This was the only known limiting factor in the assays of the activity. The synthesis of new enzyme during the substrate-induced adaptation was supported by Lee and Williams' demonstration that the increase of TPO activity in animals given tryptophan was prevented by ethionine, and that this inhibition was overcome by large doses of methionine (15). The synthesis of new enzyme during the hormone-induced adaptation was also indicated, though less surely, by the lack of any effect *in vitro* of cortisone or histidine on the enzyme, and by the development of the increase *in vivo* with an effective compound during a few hours after its administration instead of immediately (7). It is possible that the enzyme is increased by the tryptophan-induced mechanism after the administration of cortisone, since cortisone increases protein breakdown and raises the level of free amino acids in the body (16–19). On the other hand, the effect of hypophysectomy, which accentuated the response of the enzyme level to hydrocortisone (6) but depressed its response to tryptophan (4, 6), appeared to indicate that different mechanisms existed for the production of the hormone- and the substrate-induced adaptations of TPO. It will be important to recognize that metabolic adaptations *in animals* can be produced in at least these two ways, regardless of the similarity or difference in the basic mechanisms responsible for the changes in enzyme levels.

SUMMARY

1. The tryptophan peroxidase activity of rat liver is lowered by adrenalectomy, and is increased in both normal and adrenalectomized animals by cortisone treatment.

2. The increase in the level of the tryptophan peroxidase activity caused by treatment with histidine and certain pharmacologically active amines can be attributed largely to the cortisone or related adrenal hormone eventually released from the animal's adrenals, either directly or following ACTH release.

3. The two ways of altering the level of this enzyme, by treatment with cortisone and certain other hormones and by administration of tryptophan, are distinguished as hormone-induced and substrate-induced enzyme adaptations, respectively.

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STUDIES OF THE NEW HAMPSHIRE CHICKEN EMBRYO

V. LIPIDES OF WHOLE PLASMA AND OF PLASMA LIPOPROTEINS*

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(Received for publication, September 27, 1954)

Earlier work in this laboratory (1) has shown that low and medium density lipoproteins ($S_f > 50$, S_f 0 to 50) predominate in the circulation of the chicken embryo of 10 to 18 days incubation. However, between 18 and 21 days a sudden large increase in higher density lipoproteins (1 to 3 S) takes place. These dense lipoproteins persist in comparable concentration in stages up to and including the adults of both sexes. Analyses for total nitrogen and total lipide reveal various changes in the internal composition of all three lipoprotein groups in the course of normal development, as well as when such abnormal experimental conditions as fasting and x-irradiation are superimposed.

The present step in the development of this study is a more detailed analysis of the lipides of whole plasma and of these lipoproteins. Amounts of sterol ester, triglyceride, unesterified sterol, and phospholipide have been determined for several embryonic stages, the 6 week chick, and the laying hen.

Materials and Methods

Plasma was from New Hampshire chickens. For each experiment with embryos blood was pooled from at least twenty-four birds. The plasma of each laying hen was analyzed separately.

Three density groups of lipoproteins were analyzed for lipides. To obtain the lightest lipoproteins (Lipoprotein Ia) the unadulterated plasma was placed in a partition cell (2) which consists essentially of four plastic anular disks sealed together with a rubber base stop-cock grease. After centrifugation at 80,000 $\times$ g for 16 hours, the top quarter contained only those lipoproteins which floated at a medium density of 1.00. The fraction was isolated by slipping this section off the column. To obtain the medium density group of lipoproteins (Lipoprotein Ib) the density of the plasma in the remaining three-quarters of the partition cells was increased to 1.07 by addition of solid KBr (pH 7.7), and the plasma was transferred to a new partition cell. Recentrifugation for 16 hours floated Lipoprotein

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Ib into the top quarter of the partition cell. The densest lipoproteins (Lipoprotein II) were obtained by increasing the density of the residual plasma to 1.21 (pH 7.7) and centrifuging at 80,000 × *g* for 16 hours.

Total lipide of these fractions and of whole plasma was obtained by an extraction procedure described by Delsal (3). 15 volumes of a 1:4 solution of methanol in methylal were added to plasma. After evaporation of the filtrate under nitrogen at room temperature, the residue was extracted with three portions of petroleum ether (b.p. 60–70°). This procedure was compared to those of Bloor (4) and MacFarlane (5) and was found in each instance to give a larger "total lipide" yield. Multiple reextractions of the protein residue after extraction by the method of Delsal produced no further lipide nor did reextraction with hot ethyl alcohol yield additional material. Tests for total phosphorus (6, 7) indicated that practically all the phospholipide had been removed from the protein. Spectrophotometric analyses of the extracted lipide revealed negligible oxidation of the fatty acids.

Separation of the total lipide into constituent classes was achieved by use of the silicic acid chromatographic technique of Fillerup and Mead (8). The *sterol ester* fraction was eluted from the silicic acid by a solvent consisting of 1 per cent diethyl ether in petroleum ether (b.p. 60–70°). The *triglyceride* was obtained by eluting with 4 per cent ether in petroleum ether. Unesterified *sterol* was eluted with 10 per cent ether in petroleum ether, and absolute methanol served to elute the *phospholipide* fraction. Since various contaminants accompanied the crude phospholipide fraction thus obtained, the residue remaining after evaporation of the methanol was reextracted with petroleum ether. Even so, this fraction was considered to be only approximate, since certain lipide-soluble substances may also have been present.

After separation, the various lipide fractions were quantitatively transferred to 1 ml. beakers. This was accomplished by evaporating from two 100 ml. beakers the eluting solvents at room temperature, washing the lipide residue with eight 2 ml. aliquots of petroleum ether to the bottom of a single beaker, evaporating again, and transferring the concentrated lipide to a 1 ml. beaker with five or six 0.5 ml. aliquots of petroleum ether, each aliquot being evaporated before addition of the next. After the lipide was dried under nitrogen and weighed to the nearest 0.1 mg., the value obtained was converted to mg. per ml. of whole plasma.[1]

Several verifying tests were applied to the fractions. The *sterol ester*

[1] When duplicate separations were made of the same sample, the lipide weights agreed within 5 per cent except for the sterol fraction, in which differences as high as 25 per cent were obtained because of the difficulty in transferring and weighing small amounts (1 to 2 mg.) of material.

fraction was crystalline. Liebermann-Burchard reactions were run on it, colorimetric comparisons being made with a cholesterol oleate standard. Within experimental error the fraction consisted entirely of sterol ester. No phosphorus was detectable (6, 7), nor was free fatty acid present as indicated by titration with NaOH.

The *triglyceride* fraction was a colorless to pale yellow oil. Sterol was absent, as shown by the Liebermann-Burchard test. No free fatty acids were present.

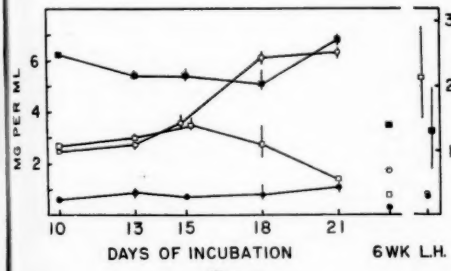

FIG. 1

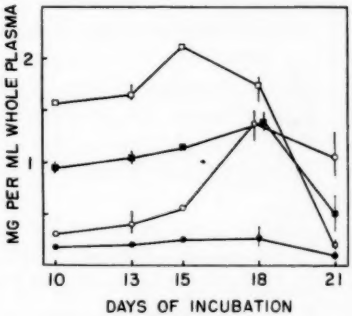

FIG. 2

FIG. 1. Lipides of whole plasma. ■, phospholipide; □, triglyceride; ●, sterol; ○, sterol ester. Vertical lines through the points indicate the range. Each point represents the mean value of two to four experiments. For each analysis, except for the laying hens, plasma was pooled from at least twelve birds. Individual determinations were made on three hens.

FIG. 2. Lipides of Lipoprotein Ia. Symbols as for Fig. 1. Each point represents the mean value of three or four experiments. Plasma was pooled from at least twenty-four embryos for each analysis.

The *sterol* fraction was crystalline. Liebermann-Burchard analyses with cholesterol standards indicated that it was nearly pure sterol. No free fatty acids were present.

The *phospholipide* fraction was waxy in appearance. The results of twelve analyses for total phosphorus in samples from different lipoproteins and at different stages indicated that the material was 85 to 95 per cent phospholipide. No sterols or free fatty acids were present in the fraction. A small amount of carotinoid was present.

Results

The amounts of the various lipide constituents in whole plasma varied considerably with the embryonic stage (Fig. 1). Although phospholipide was present at a relatively high level throughout development, it decreased significantly from the 10th to 18th day of incubation and increased sud-

denly between the 18th and hatching days. Triglyceride and sterol ester of whole plasma were present in practically identical quantities until the 15th day of incubation, after which a rise in sterol ester occurred, continuing to the 21st day; a decline in triglyceride of similar magnitude took place during this time. Non-esterified sterol was present in low concentration in all of the embryos and rose slightly with increasing age.[2]

The four major classes of lipides were distributed among the three density groups of lipoproteins (Figs. 2, 3, and 4). The lipide components of Lipoprotein Ia varied as a function of age in respect to both total amount and predominant type (Fig. 2). The highest concentration of total lipide

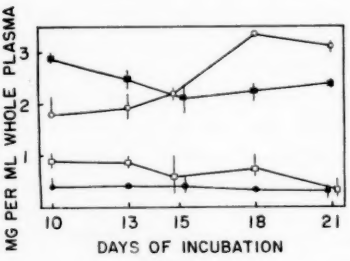

FIG. 3

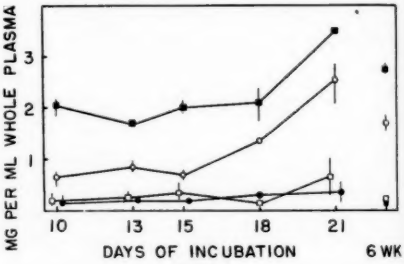

FIG. 4

FIG. 3. Lipides of Lipoprotein Ib. Symbols as for Fig. 1. Each point represents the mean value of three experiments. Plasma was pooled from at least twenty-four birds for each analysis.

FIG. 4. Lipides of Lipoprotein II. Symbols as for Fig. 1. Each point represents the mean value of two or three experiments. Plasma was pooled from at least twenty-four birds for each determination except in the case of the chicks 6 weeks old, when blood was pooled from six birds in one experiment and from twelve in another.

occurred in the plasma of the 18 day embryo. From the 10th to the 18th day of incubation, triglyceride was the predominant lipide, but from the 15th day there was a steady increase in the proportion of sterol ester until, in the newly hatched chick, it greatly outweighed triglyceride. The proportion of phospholipide in this lightest group was approximately 30 per cent.

Lipoprotein Ib was characterized by a high proportion of both sterol ester and phospholipide (Fig. 3). In the 10 and 13 day stages, phospholipide was predominant. At 15 days the concentration of these substances

[2] The amounts of triglyceride and phospholipide in the plasma of the individual hens varied considerably, while the non-esterified sterol and the sterol ester were present in consistent amounts (Fig. 1). However, the *ratios* of triglyceride to phospholipide were very similar in the hens studied.

was approximately equal. Thereafter, sterol ester was predominant.[3] The concentration of triglyceride was low in all age groups. The total lipide of this fraction was greatest in the 18 day embryo, when it amounted to 35 per cent of the total plasma lipide.

Lipoprotein II was especially rich in phospholipide (Fig. 4), and, when its concentration nearly tripled just prior to hatching, a corresponding rise was seen in the phospholipide of whole plasma (Fig. 1). A rather sharp rise in the sterol ester of this fraction was observed in the 18 day embryo.

DISCUSSION

At the present stage of investigation, the significance and origin of many of the changes in lipoprotein composition and concentration which accompany development remain obscure. Such factors as nutrition, availability of carrier material (protein), lipide utilization and synthesis play rôles in the determination of the observed spectrum which are difficult to evaluate without further experimental evidence.

These lipide-protein combinations were not centrifugation artifacts. Comparison between constituents of whole plasma and those of centrifugal fractions by such means as electrophoresis (9) and ionophoresis (1) revealed no differences attributable to the centrifugation procedure.

An exception to the rule that triglyceride was the predominant lipide of the lightest lipoproteins was observed in the 21 day embryo. Even though these light lipoproteins of the embryo contained a large amount of sterol ester and were notably deficient in triglyceride, they produced turbidity of the plasma. It is, therefore, apparent that under certain conditions lipides other than triglycerides can appear as large globules (chylomicrons) in the blood.

As early as the 15th to 18th day of incubation the amount of triglyceride in the whole plasma had begun to fall significantly, whereas the concentration of sterol ester had risen sharply (Fig. 1). Between the 18th and 21st days of incubation an even more precipitous drop in the tryglyceride content of the blood was observed, with concomitant increases in *both* sterol ester and phospholipide. These phenomena occurred concurrently with the increase in the size of the liver, which is very small until the 14th day, when its rapid growth begins. Entenman, Lorenz, and Chaikoff (10) found that the liver of the newly hatched chick contained large amounts of cholesterol, mainly as ester. (Up to 9.7 per cent of the total dry weight was cholesterol which was about one-half the total lipide.) Mc-

[3] Subsequent studies have shown that, whereas chicken embryos of more than 15 days have two major components in the Lipoprotein Ib group, the younger embryo has none or little of the lighter of these components. The lighter component contains primarily sterol ester, whereas the heavier contains mostly phospholipide.

Menamin indicated that the concentration of sterol ester in the plasma of the chicken embryo is related to the concentration of sterol ester in the liver; when the output of thyroxine in the chicken embryo of 18 to 21 days was reduced, the concentration of sterol ester in the liver went down, while that of the blood rose proportionately.[4]

It should be emphasized that such a high concentration of sterol ester in the plasma appears to be unique to the late embryo. These proportions were found neither in the adult hen (Fig. 1) nor in mature human beings (8). The significance of large amounts of sterols in the blood of the chick embryo is not known. That the phenomenon may apply to other breeds of chickens and to other species is suggested by preliminary findings on the white Leghorn embryo and the advanced rabbit fetus.

The increased incorporation of fatty acids into phospholipide between the 18th and 21st days was correlated with a large increase in Lipoprotein II which took place during this time (Fig. 4). The appearance of fatty acids in the dense lipoproteins followed fairly soon the feeding of C^{14}-labeled methyl oleate[5] to newly hatched chicks. About 40 per cent of the total radioactivity of the plasma lipide (as observed 6 hours after feeding) was in the dense fraction.

SUMMARY

The plasma lipoproteins of chicken embryos in various stages of development were divided into three groups on the basis of their flotation in aqueous media of different densities. Amounts of sterol ester, triglyceride, unesterified sterol, and phospholipide associated with each of those fractions were determined by means of a chromatographic procedure.

In whole plasma, phospholipide was present in relatively large amounts throughout development, but decreased to a measurable degree between the 10th and 18th days of incubation and then increased. A sharp rise in sterol ester began at the 15th day of incubation, with a correlating decrease in triglyceride.

The lightest lipoproteins were characterized by a predominance of triglyceride until close to the time of hatching, when sterol ester became the major lipide component of this group.

The median density group of lipoproteins contained phospholipide as the major component early in development, but a shift to sterol ester occurred after the 15th day of incubation.

The lipides of the densest lipoproteins were composed of over 50 per cent phospholipide, but increasing amounts of sterol ester appeared as development proceeded.

[4] Personal communication from J. W. McMenamin (1954).

[5] Prepared in this laboratory by Dr. Judd C. Nevenzel of the Tracer Synthesis Section, Biochemistry Division.

Much of the technical work for this study was performed by Miss Phyllis Rapaport.

The author also gratefully acknowledges the expert advice and assistance of Dr. James F. Mead, Miss Dorothy Fillerup, Dr. David Howton, and Dr. Judd C. Nevenzel.

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THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

VIII. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF L-MALATE AT pH 4 TO pH 7

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When excised tobacco leaves are cultured for 48 hours in darkness in solutions of L-malate, more malic acid is taken up and a greater proportion of the acquired acid is metabolized if the solution is supplied at pH 5 than is observed if the solution is adjusted to pH 6 (1, 2). Nevertheless, the formation of citric acid was found to be stimulated to a greater extent at pH 6 than at pH 5. When succinic acid is furnished to the leaves under the same conditions, approximately 90 per cent of the acid taken up disappears as such, apparently because of its ready convertibility to malic acid, but again the uptake is greater at pH 5 and the stimulation of the formation of citric acid is greater at pH 6. It has accordingly seemed desirable to examine the behavior of leaves cultured in solutions of L-malate adjusted to a broader range of pH in the hope that, under more extreme conditions, the response of the enzyme systems might be such as to throw some light upon the nature of the reactions that occur.

EXPERIMENTAL

The plants of *Nicotiana tabacum*, var. Connecticut shade-grown, were raised in the greenhouse in the season of 1952 and were sampled by the statistical method (3) 57 days after being set out. The coefficient of variation of the fresh weight of the ten samples (twenty leaves each) was 2.9 per cent, which is somewhat higher than is usually found, but that the samples were initially closely alike in composition is shown by the coefficient of variation of the total nitrogen content which was only 1.2 per cent.

A control sample was at once dried for analysis and another was cultured for 48 hours in water in the dark. Of the remaining samples, four were cultured for 24 hours and four for 48 hours in darkness in 0.2 M solutions of potassium L-malate adjusted respectively to pH 4, 5, 6, and 7. All samples increased slightly in fresh weight and there was no evidence of damage to the tissues.

The experimental technique has been described in previous papers and details of most of the analytical methods used are to be found in a recent

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bulletin from this Station (4). Malic acid was determined by a modification of the ion exchange chromatographic method of Busch, Hurlbert, and Potter (5), and citric acid as described by Hargreaves, Abrahams, and Vickery (6) in the pooled citric acid fractions from the column because of the presence of a significant proportion of a second as yet unidentified component in these fractions. The total organic acids were estimated from the sum of the titrations of all of the fractions from the column together with the separately determined oxalic acid which is not eluted under the conditions adopted.

The effect of the pH of the culture solution upon the uptake of potassium by the leaves is shown in Fig. 1, *A* in which data obtained by two completely independent methods are plotted. The solid lines show the increase in the alkalinity of the ash (*i.e.*, the increase of potassium carbonate) over that of the control sample, the broken lines the increase in potassium as determined in water extracts of the dried tissues by the flame photometer.[1] The control sample before culture contained 326 m.eq. of alkalinity of ash and 146 m.eq. of potassium per kilo of initial fresh weight of the leaves and there was no significant change after culture in water for 48 hours.

The effect of culture upon the total organic acidity is shown in Fig. 1, *B*; the initial sample contained 250 m.eq. per kilo. Fig. 1, *C* and Fig. 1, *D* show the accumulation of malic acid from an initial level of 173 m.eq. per kilo and the resulting stimulation of the formation of citric acid. The sample cultured in water lost 3 m.eq. of malic acid in 48 hours but increased in citric acid content from an initial level of 18 m.eq. to 26 m.eq. per kilo. However, in the leaves artificially enriched with malic acid, the formation of citric acid was stimulated from nearly 4- to more than 5-fold according to the pH of the culture solution. The greatest increase occurred at high pH.

The data for the uptake of malic acid in Fig. 2, *A* were calculated from the means of the respective values for the increase in alkalinity of ash and for the increase in potassium shown in Fig. 1, *A* on the assumption that the mean of the results of the two analytical methods, one of which is absolutely specific, provides the most reliable information. The divisors used at pH 4, 5, 6, and 7 were, respectively, 0.42, 0.70, 0.94, and 0.99, these being the fractions of the malic acid which are neutralized at the indicated acidities as calculated from a plot of the dissociation curve of malic acid.[2] Although the results at pH 5 and 6 are not consistent in trend with

[1] We are indebted to Mr. W. H. Mathis of the Department of Analytical Chemistry of this Station for these determinations.

[2] The use of these quantities involves the assumption that the potassium ion and the two ionic species and the uncharged molecules of malic acid present in the culture

earlier data, it is clear that considerably more malic acid is taken up in 48 hours at the lowest pH than at the highest.

The quantity of malic acid metabolized (Fig. 2, *B*) was calculated as the difference between the uptake and the actual increase found by analysis.

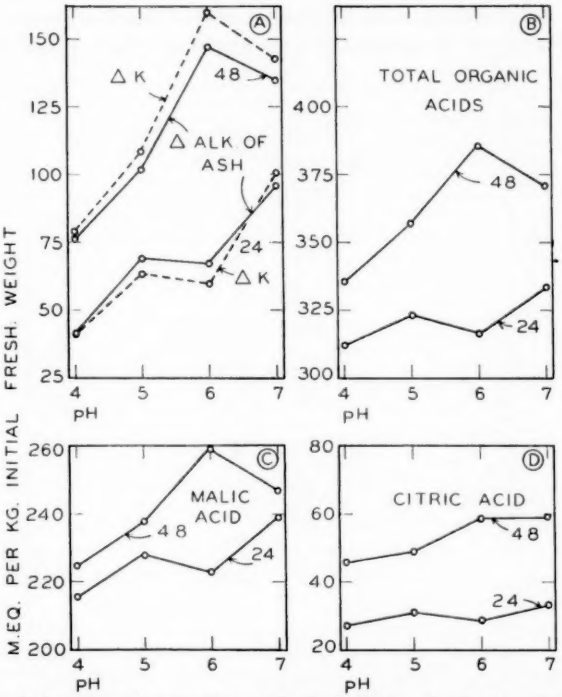

FIG. 1. *A*, increase of potassium and of alkalinity of ash (in milliequivalents per kilo of initial fresh weight) during culture of excised tobacco leaves for 24 and 48 hours in solutions of potassium L-malate at reactions from pH 4 to pH 7; *B*, effect on total non-volatile organic acids; *C*, effect on the content of malic acid; *D*, effect on the content of citric acid.

Except for the value at 24 hours at pH 6, the data yield smooth curves, which suggest that approximately twice as much malic acid was converted

solutions entered the cells in the ratio in which they occurred at the pH to which the solutions were adjusted. The validity of this assumption cannot be easily demonstrated with a metabolically active organic acid, but it appears to be true for a metabolically inactive acid such as (+)-tartaric acid at pH 5 and 6 (7). The figure used for the solution at pH 4 is probably less reliable than the others.

into other metabolic products at pH 4 as at pH 7, and the observations at pH 5 and 6 conform in trend with the results of the earlier experiment (2). The same data plotted in Fig. 2, *C* as percentages of the uptake at each point show that not only a greater amount but a greater proportion of the available malic acid was metabolized at low than at high pH, and again the data conform with previous results. However, there was little difference between the percentages metabolized at pH 6 and pH 7, and

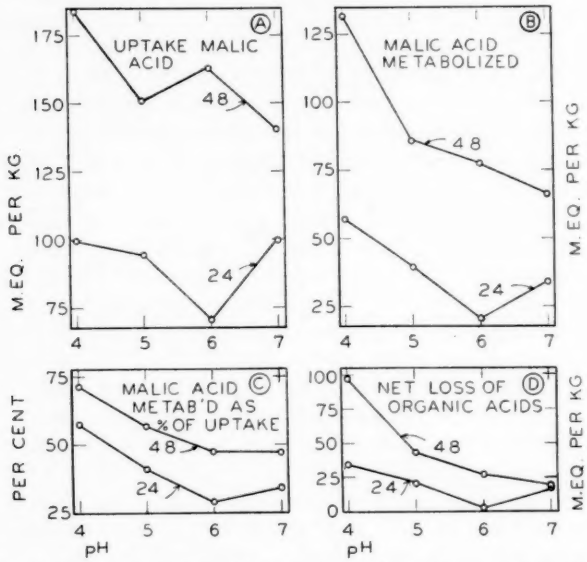

FIG. 2. *A*, uptake of malic acid by excised tobacco leaves cultured for 24 and 48 hours on solutions of potassium L-malate at reactions from pH 4 to pH 7; *B*, quantity of malic acid metabolized; *C*, malic acid metabolized as a percentage of that taken up; *D*, net loss of organic acids from the system.

there is a possibility that the value for 24 hours at pH 6 is somewhat out of line.

Fig. 2, *D* shows the differences at each point between the uptake of malic acid and the increase in total titratable organic acids, this being interpreted as the net loss of organic acid from the several systems. The utilization of acids in the general metabolism is obviously greatly stimulated at low pH, although whether the acids that disappeared were completely oxidized, as seems most likely, or were converted in whole or in part into non-acidic substances does not appear from the data.

Uptake of Solution and Transpiration Loss—Inasmuch as the culture

solutions were 0.2 M, the volume taken up per kilo of leaves can be calculated from the uptake of malic acid. If the increases in fresh weight (mean values, 18 gm. per kilo in 24 hours, 33 gm. in 48 hours) of the samples are deducted, an estimate is furnished of the transpiration of water from the leaves during the period of culture, the further correction for the loss of organic solids by respiration being negligible. The data in Table I, Line 1, are somewhat irregular but suggest that the transpiration may have been

TABLE I

Effect of Culture in 0.2 M Solutions of Potassium L-Malate upon Composition of Excised Tobacco Leaves

Data expressed in terms of initial fresh weight of 1 kilo per sample.

Line No.	Control before culture	Change in 48 hrs. in darkness				
		Water	Malate, pH 4	Malate, pH 5	Malate, pH 6	Malate, pH 7
1. Transpiration, *ml.*			420	320	360	310
2. " per single leaf, *ml.*			6.6	5.0	5.7	5.2
3. Protein N, *gm.*	2.34	−0.07	−0.13	−0.23	−0.11	−0.33
4. Inorganic solids, *gm.*	17.9	+0.1	−6.4	+8.5	+10.9	+9.8
5. Organic solids, *gm.*	74.9	−2.4	+0.0	−0.9	+1.2	+0.6
6. " " corrected for CO_2 of ash, *gm.*	82.1	−2.3	+1.7	+1.4	+4.5	+3.6
7. pH of extract of dry tissue	5.32	+0.08	+0.07	+0.10	+0.22	+0.20
8. Starch, *gm.*	2.1	−1.9	−2.0	−2.0	−1.8	−2.0
9. Glucose, *gm.*	1.97	−0.71	−1.24	−0.76	−1.07	−0.99
10. Net change organic acids (as malic), *gm.*		+1.2	−6.5	−2.9	−1.80	−1.25
11. Sum of Lines 8, 9, and 10, *gm.*		−1.3	−9.7	−5.7	−4.7	−4.2
12. Observed respiration loss, *gm.*		−2.4	−10.6	−8.7	−6.5	−5.8
13. Loss accounted for, %		54	92	65	72	72

stimulated at the lowest pH of the culture solution. The average value per single leaf (Table I, Line 2) was 5.6 ml. in 48 hours or 35 per cent of the fresh weight (16 gm.) of one leaf. The average total uptake of solution was 6.1 ml. per single leaf or 38 per cent of its fresh weight. These figures furnish an indication of the order of magnitude of the phenomena involved. It may perhaps be pointed out that the success of the method employed in these experiments for introducing metabolites into leaves is to a considerable extent dependent upon establishing conditions such that the transpiration attains useful magnitudes.

Effect on Protein Metabolism—The data for protein nitrogen are shown in Table I, Line 3. As in previous experiments (2, 8), a marked stimulation

of the decomposition of protein was observed as a result of culture of the leaves on salts of organic acid.

DISCUSSION

Conversion of Malic Acid into Citric Acid—When tobacco leaves are cultured in water or inorganic salt solutions in darkness, malic acid decreases and citric acid increases, although there is little change in the total organic acidity. Some years ago (9), upon purely analytical evidence, the hypothesis was advanced that reactions occur under these conditions by which malic acid is converted into citric acid. More recently, Zbinovsky and Burris (10) have obtained evidence from experiments in which malic acid labeled with radioactive carbon was infiltrated into tobacco leaves

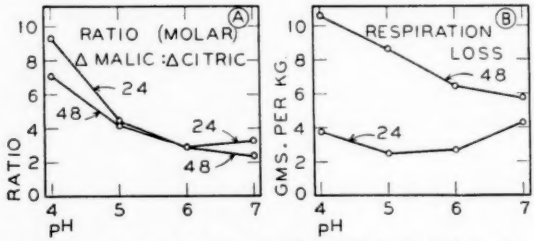

FIG. 3. *A*, molar ratio of the malic acid metabolized to the increase of citric acid in 24 and 48 hours as a function of pH of the culture solution; *B*, loss of organic solids through respiration during 24 and 48 hours of culture as a function of the pH of the culture solution.

that such an interconversion does in fact occur. Furthermore, consideration of the data obtained in a number of experiments (1, 2) in which tobacco leaves were cultured in water, in inorganic salts, or in malate led to the suggestion that 2 moles of malic acid are used up during the synthesis of 1 mole of citric acid, for in many cases the molar ratio of the malic acid which disappeared from the system to the citric acid produced approximated to 2.0. Most of the available data lie in the range between 2 and 3 and consideration must be given to the possibility that some of the malic acid may have had a fate other than transformation to citric acid.

The data plotted in Fig. 3, *A* throw some light upon this matter. The ratios of the number of moles of malic acid metabolized to the number of moles of citric acid produced at each point of observation fall along smooth curves that suggest that this ratio is a sensitive function of the pH to which the culture solution is adjusted. At low pH, where the uptake of malic acid is maximal and the proportion of the acquired acid which disappears from the system is also maximal, the molar ratio is high, being of the order

of 7 for the 48 hour period. At neutral pH, where the uptake is appreciably less and the proportion metabolized is much smaller, the ratio falls almost to 2. Fig. 1, *D* shows that citric acid accumulates to the greatest extent at neutral pH. It is accordingly possible to conclude that a sequence of reactions whereby 2 moles of malic acid are used up during the formation of a single mole of citric acid represents the predominant fate of the malic acid that disappears when this substance is supplied in culture solution in the range pH 6 to 7. At more acid pH, it is clear that a substantial part of the malic acid which disappeared had some other metabolic fate. The fact that malic acid accumulates as such to the greatest extent in the range pH 6 to 7 (Fig. 1, *C*) correlates well with this view of the situation.

Respiration Loss—Table I shows in Line 5 the changes in organic solids which occurred during culture of the leaves for 48 hours. These quantities have been corrected in Line 6 by the addition of the respective weights of the carbon dioxide, computed from the increases in the alkalinity of the ash, on the assumption that this represents carbonate derived from the combustion of the acquired potassium malate. A closer estimate of the actual losses of organic substance from the respective systems is thus provided by calculating the difference between the uptake of malic acid at each point and the observed change in corrected organic solids. These differences are plotted in gm. per kilo in Fig. 3, *B* as the respiration loss. Although the loss of organic solids during 24 hours was not greatly affected by the pH of the culture solution, the loss in 48 hours follows a smooth curve that slopes downward from somewhat more than 10 gm. per kilo at pH 4 to about 6 gm. at pH 6 and 7. The range is sufficient to give some confidence in the validity of the results, and it may be inferred that respiration, as measured by the loss of organic solids, is stimulated at low pH of the culture solution.

Lines 8 to 11 in Table I give the data for the losses, respectively, of starch, glucose, organic acids (arbitrarily calculated as malic acid), and the sums of these losses and, in Line 13, the calculation of these sums as percentages of the observed losses of organic solids through respiration (Line 12). Although in experiments in which tobacco leaves were cultured in tartaric or succinic acid salts (2, 7) at pH 5 and 6 it was possible to give a moderately satisfactory account of the losses of organic solids in terms of these three components, it was not possible to do so in the previous work in which malic acid was furnished to the leaves; nor can this be done in the present case. At pH 4, at which a large proportion of the acquired malic acid appears to have been completely removed from the system, presumably by respiration, the accounting reaches the moderately satisfactory figure of 92 per cent, but, at the less acid reactions of the culture

solution, it is clear that other components of the leaves in addition to starch, glucose, and organic acids must have made a significant contribution. Neither sucrose nor unfermentable reducing carbohydrate could have been involved, for the sucrose increased slightly and the unfermentable carbohydrate was essentially unchanged. The poor accounting in the control leaves cultured in water should not be emphasized since the observed respiration loss was small and it is extremely doubtful that the organic acids actually increased as the analysis of the samples indicates. A small difference between two samples has here had a large apparent effect and, if the change in the total organic acids is neglected, the accounting is complete.

The respiration loss in 48 hours when the culture solution was supplied at pH 4 was 4.6 gm. per kilo greater than the loss when the solution was at pH 6. Reference to Fig. 2, *D* shows that there was a correspondingly large change in the net loss of organic acids from the leaves in the same range of pH. The observed difference was 78 m.eq. which, calculated as malic acid, amounts to 5.2 gm. Although the approximate agreement between these quantities may be to some extent fortuitous, the evidence indicates that the substance which contributed a substantial part of the increased respiratory loss at low pH was malic acid acquired from the culture solution.

This result makes it possible to suggest what is doubtless an oversimplified but nevertheless reasonable explanation of the effect of the pH of the culture solution upon the ratios plotted in Fig. 3, *A*. It may be assumed that the acquired malic acid has two major metabolic fates. Some of it contributes to the respiratory loss, some to the synthesis of citric acid. The relative proportions vary with the pH of the culture solution; at low pH, where the uptake is high and the concentration of malic acid in the cells is unusually increased, much of the acid is apparently completely oxidized;[3] at pH 6 to 7 where the uptake is less, much and probably most of that part which disappears is used for the synthesis of citric acid.

It is tempting to speculate that one is here dealing with the metabolic fates of the two ionic forms in which the malic acid finds its way into the leaf cells. At pH 4, approximately 71 per cent of the malic acid in solution is present as the monovalent acid malate ion (approximately 23 per cent is uncharged); at pH 6, 89 per cent is present as the divalent ion. How-

[3] It should perhaps be pointed out that any part of the metabolized malic acid which was converted into oxalacetic or pyruvic acid would escape detection. These components would be in part, at least, decomposed during the drying of the leaf tissue, and any that remained would be decomposed when the fractions eluted from the column with formic acid were evaporated to dryness in preparation for titration. Whether or not either of these acids is present in more than trace quantities in tobacco leaf tissue under any circumstances is a matter for future investigation.

ever, questions of the permeability of the cells within the leaf structure to these respective ionic forms at once arise as well as the awkward fact that little or nothing is known of the distribution of pH within the living cells themselves or of the location within the cells at which the chemical changes occur. Extracts of the dried tissues show only minimal changes in pH as a result of the treatment of the leaves with culture solution (Table I, Line 7) but such observations furnish only a sort of average which, although it may be valid over the entire mass of tissue after this has been killed and dried, tells little about the pH at which the actual metabolic event took place during the culture period. If a culture solution at pH 4 or at pH 7 enters a cell, the vacuole content of which is maintained at close to pH 5.3, the distribution of the ions must immediately become adjusted to the new physicochemical condition. Accordingly, speculation on the metabolic fate of specific ions after they have entered the cell must be founded on distinctly dubious assumptions.

Another view of the situation is to suppose that more malic acid is used up at pH 4 than at pH 6 or 7 simply because more was taken up, that is to say that the law of mass action was the controlling influence. This view cannot account for the observations at the end of 24 hours of culture, for in this period the uptake (Fig. 2, *A*) was little influenced by the pH of the culture solution, being close to 100 m.eq. in three of the four cases. Nevertheless, 57 per cent of the acquired malic acid was metabolized at pH 4 and only from 29 to 34 per cent at pH 6 and 7 (Fig. 2, *C*). In 48 hours, the uptakes were 184 m.eq. at pH 4 and 140 m.eq. at pH 7, and the amounts metabolized were, respectively, 132 and 67 m.eq.; *i.e.*, 72 and 47 per cent of the uptake. Thus, over this range of pH, a decrease in uptake by one-quarter is correlated with a decrease in utilization by one-half.

In any case, it would seem reasonable to assume that the chemical behavior of the system represents the response of the cells to the problem of maintaining an internal pH consistent with their own integrity. The advent during 48 hours of a quantity of culture solution of the order of one-third of the weight of the cell and buffered at pH 4, a reaction which is considerably more acid than the fluids of the leaf (approximately pH 5.3), would presumably call forth chemical changes which would tend to diminish this surplus of acidity. The observation is that disappearance of malic acid is stimulated. When the culture solution is supplied at pH 6 or 7, on the other hand, the problem is to deal with an excess of alkali; accordingly the complete oxidation of organic acids is minimized as shown by the flattening of the curve for net loss (Fig. 2, *D*), and there is a stimulation of the conversion of malic acid into citric acid by which, if the molar ratio is taken into account, four carboxyl groups are replaced by three, an outcome which is more favorable than the loss of all four carboxyl groups.

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Since the pH of the tissue extract becomes only about 0.2 unit more alkaline, it is quite possible that still other buffering effects as yet undetected come into play.

Comparison with Effect of Culture in Citrate—In previous experiments (8) in which tobacco leaves were cultured in solutions of citrate under conditions similar to those in the present study, it was observed that the uptake of citric acid was greater at pH 4 than at pH 6 (the results at pH 7 differed little from those at pH 6) and that the amount of citric acid which disappeared from the system was greater at low pH. The uptakes at pH 4 and 6 were, respectively, 230 and 183 m.eq. per kilo, and the amounts metabolized were 155 and 71 m.eq. The loss of organic acids from the system, presumably through respiration, was also greatest at pH 4. Accordingly, the main effects of the pH of the culture solution upon the observed behavior were essentially the same with either acid. The outstanding difference in the two instances is in the effect upon the reaction or series of reactions whereby malic acid is converted into citric acid. As pointed out above, this chemical change is promoted and rendered more efficient (*i.e.*, the molar ratio diminishes and approaches 2) as the pH of the malic acid culture solution becomes less acid. When citrate is supplied, on the other hand, the reaction is reversed, for under these conditions malic acid *increases*. Furthermore, provided that sufficient citric acid enters the leaves, the increase of malic acid does not appear to be greatly affected by the pH of the culture solution. However, additional study would be required before it could be concluded that there is no pH effect; the available evidence merely suggests that it is small.

Grateful acknowledgment is made to Marjorie D. Abrahams, Katherine A. Clark, and Laurence S. Nolan for technical assistance, to Dr. Israel Zelitch and Dr. James K. Palmer for helpful discussion, and to the National Science Foundation for a grant which supported a part of the expense of this investigation.

SUMMARY

Excised leaves of tobacco (*Nicotiana tabacum*, var. Connecticut shade-grown), when cultured in 0.2 M solutions of potassium L-malate, take up more malic acid from a solution adjusted to pH 4 than from a solution at pH 6 or 7; furthermore, a larger proportion of the acquired malic acid is metabolized at low than at neutral pH. On the other hand, the stimulation of the formation of citric acid is greater at the higher pH. The increased loss of malic acid from the system at low pH is attributed to increased respiration of the acquired acid.

Although other views of the situation are possible, the behavior is most

simply accounted for by the assumption that the increased respiration of organic acids at low pH represents the response of the cells to the advent of a solution more acid than the pH of the fluids within the cell. When the culture solution is supplied at a reaction more alkaline than the cell solution, loss of acid components by respiration is minimized and the conversion of malic acid into citric acid is stimulated, this pathway of metabolism being more advantageous than complete oxidation of the acid. Malic acid also accumulates as such.

Addendum—It has recently been noticed that, if the differences between the uptake of acid (Fig. 2, *A*) and corresponding increase in total organic acids (Fig. 1, *B*) are deducted from the respective quantities of malic acid metabolized (Fig. 2, *B*), an estimate is obtained of the amount of acquired acid which in each sample is converted into a different organic acid. The assumption involved is that the loss of organic acids from each system represents decarboxylation reactions that fell exclusively upon the malic acid taken up. The molar ratios of the amounts of malic acid, that were converted to a different acid in 48 hours, to the corresponding increases in citric acid were respectively 1.8, 2.1, 1.9, and 1.7 at pH 4, 5, 6, and 7. Accordingly, this ratio is not sensitive to the pH of the culture solution, and its constancy is more interesting in the light of the statement on p. 332 regarding the reverse reaction which occurs when citric acid is made available to the leaves in culture solution. The mean value of the ratio for the seven concordant observations (the ratio for the sample cultured for 24 hours at pH 4 was obviously out of line) was 1.99 ± 0.26.

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EXCRETION OF ESTROGEN METABOLITES BY HUMANS*

I. THE FATE OF SMALL DOSES OF ESTRONE AND ESTRADIOL-17β

By CHARLES T. BEER and T. F. GALLAGHER

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(Received for publication, November 4, 1954)

Since the isolation of estrone and estradiol and the demonstration of their relationship to the follicular phase of ovarian function, numerous investigations of the metabolism and fate of these compounds have been reported (1). While the available evidence strongly indicates that estradiol is the hormone found in ovarian follicular fluid, the interconvertibility of the two substances has been repeatedly demonstrated. As a basis for more detailed investigations, it was desired to obtain information about the metabolic transformation and elimination of these two closely related compounds by human subjects. With isotopically labeled precursors it was possible to evaluate the importance of estrone and estriol as estrogen metabolites and at the same time to examine other related transformation products. In addition, a study of the hydrolytic procedure necessary to free the hormonal metabolites from their conjugates was made. Other details of the biochemistry and physiology of these hormones such as a comparison of intravenous and intramuscular administration, the duration of the hormone and its metabolites in the human body, and the route of excretion have been examined.

The results obtained present many points of similarity as well as contrast with other steroid hormones that have been investigated in a similar way in these laboratories (2–4). In particular the difference in rate of elimination of the metabolites of the phenolic steroid hormones compared with their neutral analogue is apparent. It has been shown that, while estrone and estriol constitute a sensible portion of the radioactivity recovered in the phenolic fraction after hydrolysis of the conjugated steroids by β-glucuronidase, these two substances comprise less than half of the total metabolites in urine after a tracer dose of hormone. Other details of the transformation and elimination of these hormones will be dealt with in the discussion and in the subsequent report (5).

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EXPERIMENTAL

Intravenous Infusions—Estrone-16-C^{14} or estradiol-17β-16-C^{14} (about 0.5 mg.) dissolved in 1 to 2 ml. of absolute ethanol was poured, with brisk stirring, into 600 ml. of sterile isotonic saline.[1] The subjects were given intravenous infusions of accurately determined volumes of this solution during a 2 hour period. A known sample of the infusion solution was extracted with ether for 48 hours, and the radioactivity of the extract was determined by plating as solid mounts. The total radioactivity infused was calculated from the count of the fraction analyzed.

Intramuscular Injections—Estradiol-17β-16-C^{14} (about 0.25 mg.) in a sterile ampul was dissolved in 1 ml. of warm benzyl alcohol. 2.5 ml. of sesame oil were added, and, after sealing, the ampul was autoclaved at 140° for successive periods of 70 minutes and 90 minutes, and cooled to room temperature in the interval. An independent isotopic dilution measurement established that estradiol-17β-16-C^{14} was unchanged by this sterilization. 3 ml. of solution were injected intramuscularly as a single dose. Weighed samples of the solution not required for injection were made to volume in ethanol and known portions were counted after evaporation on planchets. From the density of the benzyl alcohol-sesame oil mixture, the radioactivity injected in 3 ml. was calculated.

Collections—Urine collections were made for successive 24 hour periods. The start of the first period coincided with the initial administration of the hormone. The urine was stored in a refrigerator prior to hydrolysis, which as a rule was started within a few hours of the end of the collection period.

Hydrolysis A—The urine was adjusted to pH 5 with sulfuric acid. 10 per cent by volume of sodium acetate buffer (76 gm. of sodium acetate and 13 ml. of glacial acetic acid made to 4 liters with water) at pH 5 was then added, followed by 300 Fishman units of β-glucuronidase[2] per ml. of urine. The mixture was incubated at 37° for 5 days and then continuously extracted with ether for 48 hours at pH 5, except in one case (Subject L) in which extraction was at pH 1.

Hydrolysis B—The urine was refluxed for 30 minutes with 5 per cent (volume per volume) of concentrated sulfuric acid and, after cooling, was extracted for 48 hours with ether.

Fractionation. Stage 1—The ether extract from Hydrolysis A or B above was concentrated to about 200 ml. and extracted with 200 ml. of 9 per cent sodium bicarbonate solution. The bicarbonate solution was extracted three more times with 200 ml. of ether, and the combined ether

[1] Estrone-16-C^{14} and estradiol-17β-16-C^{14} were purchased from Charles E. Frosst and Company, Montreal, Canada.

[2] Obtained from the Warner-Chilcott Laboratories, a division of Warner-Hudnut, Inc., New York, now commercially available under the trade name Ketodase.

extracts were back-washed twice with 20 ml. of water. The combined bicarbonate and aqueous washings were reserved as the *acidic fraction.*

Stage 2—This separation is essentially that proposed by Engel (6). The ether extract from Stage 1 was evaporated and the residue dissolved in a mixture of 100 ml. of toluene and 25 ml. of N sodium hydroxide. The aqueous base was separated and the toluene was reextracted with three further 25 ml. portions of N sodium hydroxide. The toluene was back-washed three times with 5 ml. of water, and the washings were added to the combined N sodium hydroxide extract. The toluene solution contained the *neutral fraction.*

Stage 3—The N sodium hydroxide extract from Stage 2 was acidified to pH 1 with sulfuric acid and continuously extracted for 48 hours with ether. Subject L was an exception in that the solution was extracted at pH 7 instead of pH 1. The ether extract contained the *phenolic fraction.*

Stage 4—The phenolic fraction from Stage 3 was separated into ketonic and non-ketonic components by means of Girard's Reagent T. To the fraction dissolved in 10 ml. of ethanol were added 1 gm. of Reagent T and 1 ml. of glacial acetic acid. After refluxing under nitrogen for 1 hour, the mixture was poured into 100 gm. of crushed ice and adjusted to neutrality with 2 N sodium hydroxide. The aqueous solution was extracted four times with an equal volume of ether, and the combined ether extracts were back-washed twice with 0.1 volume of water. The ether extract was dried and evaporated to give the *non-ketonic phenolic fraction.*

The combined aqueous portion and washings were treated with 0.1 volume of concentrated hydrochloric acid and continuously extracted for 48 hours with ether. The dried ether extract was the *ketonic phenolic fraction.*

Estimation of Metabolites in Phenolic Fraction. Isotopic Dilution—The general procedure was as follows: Known weights of highly purified non-radioactive estrone and estriol were added to a known fraction of the radioactive phenolic extract. The latter was then separated into ketonic and non-ketonic components by the method described for Stage 4. The ketonic-phenolic fraction was chromatographed directly on a silica gel column and eluted with petroleum ether with gradually increasing quantities of ethyl ether or ethyl acetate. The estrone fractions, characterized by infra-red spectrometry, were combined and recrystallized to constant specific activity from ethanol. The non-ketonic phenolic fraction was acetylated at room temperature with pyridine and acetic anhydride and chromatographed in a similar way on silica gel. The eluates containing estriol triacetate, as judged by infra-red spectra, were combined and hydrolyzed by refluxing for 30 minutes with 15 per cent aqueous methanolic potassium hydroxide. The estriol isolated was recrystallized to constant

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specific activity. The amount of radioactive metabolite (E) in the phenolic fraction was calculated from Formula 1.

$$E = \frac{PGZ}{L} \quad (1)$$

where P = counts per minute of the total phenolic fraction, L = counts per minute of the sample of phenolic fraction taken for analysis, G = the weight of carrier added to phenolic sample L, and Z = counts per minute per mg. of purified product.

Isotopic Analysis—A known fraction of an ethanol solution containing an extract or pure compound was evaporated on nickel-plated steel planchets. The amount of solid on the planchets was usually less than 0.3 mg. per sq. cm. The radioactivity was measured in a windowless gas flow counter and counted to ±5 per cent accuracy. The observed counts were converted to the "infinitely thin" range by the table of Yankwich and Weigl (7). Samples of low specific activity or samples liable to contaminate the gas flow counter (*e.g.* urinary acidic fractions containing benzoic acid) were oxidized by the method of Van Slyke and Folch (8), and the radioactivity of the resultant carbon dioxide was measured by the method of Eidinoff (9). The factor relating the counts obtained by this method to those obtained with solid mounts was determined experimentally by counting portions of a series of radioactive samples by both methods. Known fractions of urine specimens were dried at about 80° in a stream of nitrogen, oxidized, and the carbon dioxide counted to ±5 per cent accuracy. Feces were homogenized with water in a Waring blendor, and known fractions were removed as quickly as possible before appreciable sedimentation had occurred. The sample was dried, oxidized, and the carbon dioxide counted. Owing to difficulties in sampling the error of measurement of radioactivity in fecal samples was at least 10 per cent.

Subjects and Administration—The subjects were female patients, showing no evidence of gross abnormalities in liver or kidney function. Subject L was aged 56 years and had hypercholesterolemia xanthoma tendinosum; Subject R was 26 years old and had sarcoidosis. Subject G, 35 years of age, had carcinoma of the rectum; Subject V, 49 years old, had hypercholesterolemia xanthoma tuberosum. Subject F, 42 years old, had rheumatic heart disease; Subject M, 75 years old, had diabetes mellitus. Subjects R, G, and F were premenopausal; the others were postmenopausal. The amount and radioactivity of the dose are reported in Table I. Table I also gives the daily urinary excretion of radioactivity by the subjects and the fecal radioactivity for Subjects V and F.

Distribution of Radioactivity in Urinary Fractions (*Subjects L, R, G, and*

V)—Samples of the 1st day's urine collections from Subjects L, R, G, and V were hydrolyzed with β-glucuronidase and fractionated as described above. The distribution of radioactivity is shown in Table II. The corresponding data obtained after hydrolysis with hot sulfuric acid are also presented in Table II.

Effect of Further Hydrolysis on Acidic Fraction of Acid-Hydrolyzed Urine (Subject R)—The sodium bicarbonate extract of the ether-soluble metabo-

TABLE I

Excretion of Radioactivity in Urine and Feces after Injection of Estrone-16-C^{14} and Estradiol-17β-16-C^{14} in Women

Subject	L	R	G	V	F	M
Compound	Estrone	Estrone	Estradiol	Estradiol	Estradiol	Estradiol
Route of administration	Intravenous	Intravenous	Intravenous	Intravenous	Intramuscular	Intramuscular
Dose, *c.p.m.* × 10^{-3}	715	1088	848	895	887	720
Day	Radioactivity in urine, c.p.m. × 10^{-3}					
1st	332 (46)	362 (33)	230 (27)	311 (35)	429 (48)	159 (22)
2nd	118 (17)	187 (17)	118 (14)	148 (17)	169 (19)	153 (21)
3rd	77 (11)	65 (6)	71 (8)	68 (8)	40 (5)	53 (7)
4th	21 (3)	24 (2)	43 (5)	37 (4)	11 (1)	25 (3)
5th					6 (0.7)	17 (2)
6th					3 (0.35)	7 (1)

The figures in parentheses represent per cent of the dose.

Feces from Subject V were devoid of radioactivity on the 1st day and contained 26,000 c.p.m. on the 2nd day and 40,000 c.p.m. on the combined 4th and 5th days, representing 3 and 4 per cent of the dose respectively. No feces were excreted on the 3rd day by this subject. The fecal radioactivity (in counts per minute) of Subject F was 500 on the 1st day, 71,000 on the 2nd day, 7000 on the 3rd day, 2000 on the 4th day (0.05, 8, 1, and 0.2 per cent of the dose respectively).

lites from the acid-hydrolyzed urine of Subject R was divided into three portions. These were treated as follows:

β-Glucuronidase Hydrolysis—A known amount of the bicarbonate extract was adjusted to pH 5 with dilute sulfuric acid. Acetate buffer and β-glucuronidase were then added as described under Hydrolysis A. After 5 days incubation at 37°, the mixture was continuously extracted with ether for 48 hours, and the extract was separated into neutral phenolic and acidic fractions. The residual aqueous solution was adjusted to pH 1 with sulfuric acid, reextracted with ether, and the extract fractionated. Fig. 1 (*1*) outlines this process and gives the distribution of radioactivity in the various fractions, calculated on the basis of the total acidic fraction from the urine, *i.e.* 182,000 c.p.m.

Further Reflux with Acid—A second portion of the sodium bicarbonate

extract was adjusted to pH 1 with sulfuric acid. After adding a further 5 per cent by volume of concentrated sulfuric acid, the solution was refluxed for 40 minutes and extracted with ether for 48 hours. The extract was fractionated; the distribution of radioactivity, calculated on the basis of the total urinary acidic activity, is shown in Fig. 1 (*2*).

Control—The third portion of the sodium bicarbonate extract was adjusted to pH 5 with sulfuric acid, extracted for 48 hours with ether, and the extract fractionated. The residual aqueous solution was adjusted to pH

TABLE II

Fractionation of Radioactivity from Urine of 1st Day after Intravenous Administration of Estrone-16-C^{14} and Estradiol-17β-16-C^{14}; Comparison of Enzymatic and Acid Hydrolysis; Measurements of Estrone and Estriol by Isotopic Dilution after β-Glucuronidase Hydrolysis

Results in counts per minute × 10^{-3}.

Subject / Compound	L Estrone	R Estrone	G Estradiol	V Estradiol
Dose	715	1088	848	895
Urine	332	362	230	311
Neutral. E.	14	7	9	7
" A.	10	7	14	8
Acidic. E.	47	13	7	9
" A.	198	182	31	99
Residual urine. E.	80	84	41	51
" " A.	48	18	18	21
Phenolic. E.	172	236	160	238
" A.	53	149	147	132
Estrone. E.	34	28	17	27
Estriol. "	49	112	26	98

E., enzymatic hydrolysis with beef liver β-glucuronidase; A., acid hydrolysis (5 per cent sulfuric acid).

1 with sulfuric acid, reextracted with ether for 24 hours, and the extract fractionated. Fig. 1 (*3*) shows the distribution of radioactivity obtained by this procedure.

Details of the amount of radioactive phenolic fractions of the 1st day's urine taken for analysis, the weight of added carrier compounds, and the specific activities after isolation and purification are as follows:

Subject L—The phenolic fraction (172,000 c.p.m.) of this subject was extracted at pH 7. The sample taken for analysis had 107,000 c.p.m., and to it were added 50.9 mg. of estrone and 52.3 mg. of estriol. The specific activities of the isolated compounds were as follows: estrone 410 c.p.m. per mg., estriol 568 c.p.m. per mg.

Subject R—The total urinary phenolic radioactivity was 236,000 c.p.m.

The phenolic sample taken for analysis had 189,000 c.p.m. To it were added 56.4 mg. of estrone and 54.3 mg. of estriol. The specific activities

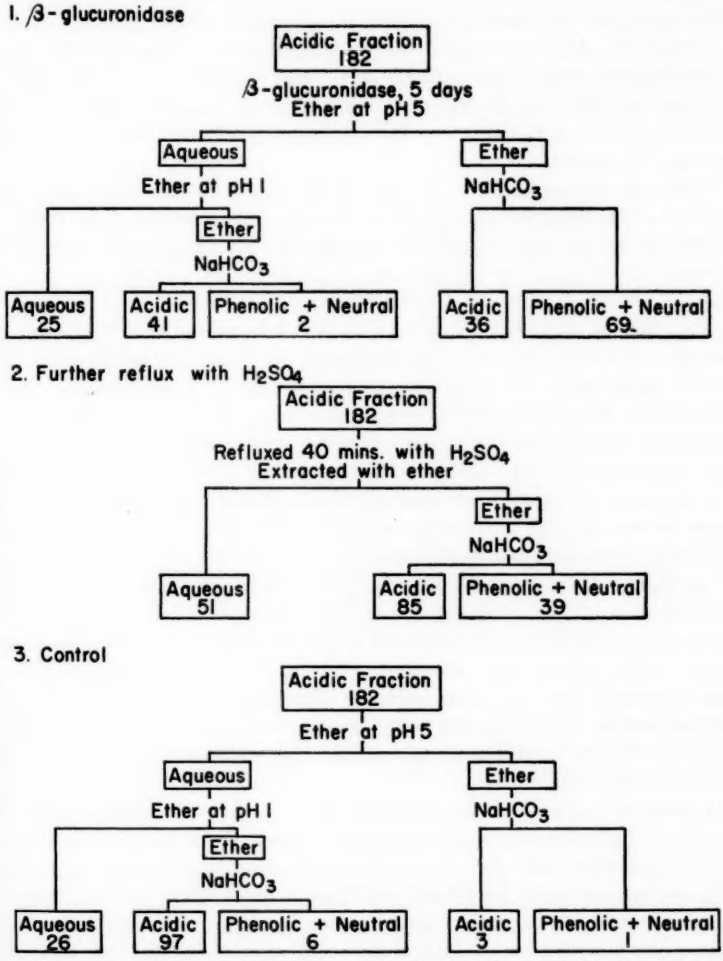

FIG. 1. Flow diagram of the further hydrolysis of the acidic fraction after acid hydrolysis; Subject R.

of the isolated compounds were as follows: estrone 398 c.p.m. per mg., estriol 1650 c.p.m. per mg.

Subject G—The total urinary phenolic radioactivity was 160,000 c.p.m.

The sample for analysis had 110,000 c.p.m., and the amount of carrier compounds added were 50.7 mg. for estrone, 50.1 mg. for estriol. When recovered and purified, the specific activities of these compounds were 231 c.p.m. per mg. for estrone, 358 c.p.m. per mg. for estriol.

Subject V—The total urinary phenolic radioactivity was 238,000 c.p.m. The phenolic sample taken for analysis had 192,000 c.p.m.; 49.1 mg. of estrone and 50.0 mg. of estriol were added, and, when isolated and purified, the specific activities were 448 c.p.m. per mg. for estrone, 1573 c.p.m. per mg. for estriol.

From the foregoing data the amount of radioactive estrone and estriol present in the total urinary phenolic fractions was calculated by Formula 1, and the results were rounded to the nearest thousand counts (Table II).

DISCUSSION

Excretion of Radioactivity after Estrogen Administration—Following intravenous or intramuscular administration of either estrone or estradiol-17β in small doses, the elimination of metabolites in the urine is a slow process by comparison with other steroid hormones. Thus on the 1st day, from 22 to 48 per cent of the radioactivity, or an average of 35 per cent of the dose administered, was found in the urine. During the 2nd day an average of 18 per cent of the dose administered was recovered in the urine, while on the 3rd day excretion averaged about 8 per cent of the dose; on the 4th day about 3 per cent of the dose was found. In Subjects M and F, in which continued measurements were made, small amounts of radioactivity were still excreted after this time. The total urinary recovery of radioactivity therefore corresponded to about 65 per cent of the hormone given. The radioactivity recovered was comparable in total amount to that obtained after the administration of neutral steroid hormones, such as testosterone and hydrocortisone, but the rate at which it appeared in the urine was slower and more prolonged. A comparison with testosterone is illustrated in Fig. 2.

A possible explanation for this difference in rate of excretion may be found in the relatively stable complex formed by estrogenic steroids or their metabolites with the proteins of serum and liver, as shown by Szego (10) and more recently by Riegel and Mueller (11). The "protein-bound" product would retard renal excretion and very probably simultaneously expose the hormone or its immediate metabolic transformation products to more extensive degradation by various enzyme systems of the animal body. This explanation would be consistent with the finding in these and other studies (12) that metabolites of estradiol-17β which are closely related chemically, such as estrone and estriol, constitute but a small per cent of the administered hormone. Additional evidence of more deep

seated transformation of the estrogens is afforded by the finding of considerable radioactivity in the non-phenolic fractions after hydrolysis of conjugates and exhaustive extraction with ether. It would appear that much of this non-phenolic radioactivity may represent hormone molecules that have been altered in other respects than in simple hydroxylation at C-16 or oxidation of the C-17 hydroxyl group during the time the hormone was in contact with organs and tissues. It is possible, of course, that con-

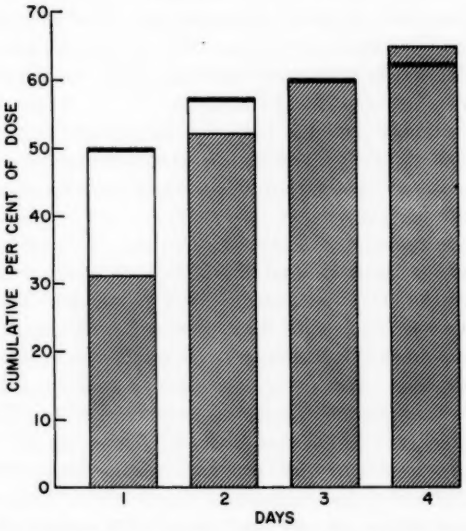

FIG. 2. Comparison of the urinary excretion of radioactivity after estradiol-17β-16-C^{14} and testosterone-4-C^{14}. The shaded areas are average values for estradiol-17β; the clear areas are average values from five comparable experiments with testosterone in men, one published (2) and four unpublished studies from these laboratories.

jugates incompletely cleaved by the hydrolytic procedures used may be responsible for the finding of radioactivity in these non-phenolic fractions. However, the studies of Riegel and Mueller (11) with rat liver homogenates indicate the formation of highly stable complexes of a metabolite of estradiol, and analogous reactions may occur in the human species. As a result, both processes, extensive degradation and the formation of as yet unrecognized conjugates resistant to the hydrolytic procedures employed, may be implied from the results.

There was no difference apparent in the excretion of radioactivity after the intravenous administration of either estrone or estradiol-17β. If these

two substances are metabolically interconvertible, the results obtained would support this view. Similarly if estradiol-17β is the true hormone and estrone is one of the first metabolic transformation products, the similarity in the excretion pattern of the two steroids is explicable. There was likewise no apparent difference in the excretion pattern of estradiol when given intravenously or intramuscularly, according to the criteria available in these studies. From the fact that essentially equal amounts of radioactivity were recovered in the urine on each day studied, the absorption of estradiol from an oily intramuscular depot over a 24 hour period must be a relatively efficient process. These results are comparable to those reported for both intravenous and intramuscular absorption of testosterone for which the excretion of metabolites was similar in amount and kind (2, 13). It may well be, however, that when studied more critically, for example at hourly intervals following introduction of the hormone, significant differences will be uncovered. Experiments of this type are in progress in these laboratories.

From the data reported and the criteria used, the physical condition of the patients was without measurable influence on the excretory pattern after administration of the hormones. Of particular significance is the fact that the two patients with hypercholesterolemia, and the associated serious disorders of lipide metabolism, were in all respects quite comparable to the other subjects. The age of the subject and the state of ovarian function similarly appeared to have no influence on the manner in which a tracer dose of hormone was metabolized. These results with the estrogens are thus in agreement with the conclusions drawn from analogous studies in patients given hydrocortisone (3), in which the excretion pattern was independent of the age, sex, and physical condition of the patients. It should be emphasized that kidney and liver function, in so far as this could be ascertained, were normal in all of the subjects. The conclusion, therefore, that the rate of excretion of hormone metabolites is not materially influenced by these diseases is important in that a variety of patients may be used for more detailed and searching investigations.

The relatively minor excretion of estrogen metabolites in the feces is suggestive that biliary and intestinal elimination play but minor rôles in the metabolism of the estrogens by human subjects. An enterohepatic circulation of the estrogens has been demonstrated in the dog (14), but the total amount of material involved in this process was small. In view of the relatively delayed urinary excretion of estrogen metabolites, the possibility that such circulation operates cannot be ignored, and further studies of bile in humans must be made. Nevertheless, as with the neutral steroid hormones testosterone and hydrocortisone, the contrast between humans and rodents is emphasized in that the fecal route of elimina-

tion for all the metabolites of these hormones is the major one in the rodent (15–17). The species difference in the route of elimination very likely reflects different metabolic alterations produced in the hormones, and the relation of these changes to the fundamentals of hormone action remains an important area for investigation.

Hydrolysis of Urinary Conjugates—It is clear from the results recorded in Table II and Fig. 1 that hydrolysis by means of beef liver β-glucuronidase is a much more efficient method for cleavage of the conjugates of the phenolic metabolites of estrone and estradiol than was the vigorous acid hydrolysis employed in the comparison studies. Thus, in three of the four examples reported, the phenolic radioactivity after enzymatic hydrolysis was almost twice that obtained after acid hydrolysis and was slightly higher in the remaining instance. A reflection of this can be seen in the acidic fraction after enzymatic and after acid hydrolysis. In each instance there was more radioactivity in the acidic fraction after acid hydrolysis than after enzyme treatment. From the evidence presented in Fig. 1, much of the radioactivity in the acidic fraction following acid hydrolysis was unhydrolyzed glucuronosides. Since the largest portion of the urinary metabolites of the estrogens appears in the phenolic fraction, hydrolysis by the enzyme β-glucuronidase is a superior procedure for these particular products. It is evident that not all of the metabolites are phenols, since a small amount of radioactivity appeared in the neutral fraction and a much larger amount remained in the urine after hydrolysis and extraction.

With the information at hand, the significance of the neutral metabolites is problematical. The amount obtained was about the same whether enzymatic or acid hydrolysis was employed, and, in the absence of further characterization, it cannot even be presumed that this material is actually neutral. The small neutral radioactivity may represent minor contamination by phenolic or even acidic materials.

The amount of radioactivity found in the residual urine, however, cannot be readily dismissed. After enzymatic hydrolysis this fraction was larger than was anticipated and may indicate conjugation with other substances than glucuronic acid. Support for this view may be found in the fact that there was a smaller amount of radioactivity in the residual urine after the less specific hydrolysis with acid. Even with this procedure a certain amount of radioactivity remained unextracted from the urine. The difference observed in the radioactivity of the residual urine after acid and enzymatic hydrolysis is sufficiently small to indicate that little additional material will be obtained by hydrolysis with acid subsequent to treatment with the enzyme. This conclusion has been confirmed by experiments recorded in the subsequent report.

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There was only a minor amount of radioactivity in the acidic fraction after enzymatic hydrolysis with the exception of Subject L. In this instance, the urine after enzymatic treatment was extracted at pH 1, whereas in the other studies ether extraction has been carried out at pH 5. Whether at the lower pH some secondary alteration, such as condensation with another urinary constituent, accelerated oxidation or the like, was responsible for the discrepancy in this instance cannot be decided on the present evidence. However, after enzymatic hydrolysis it is clear that the free phenolic metabolites are efficiently extracted at pH 5.

On the other hand when enzymatic hydrolysis was applied to the combined acidic fraction and residual urine after the urine had been boiled initially with mineral acid, there was a significant amount of radioactivity recovered in the phenolic plus neutral fraction, as shown in Fig. 1. These experiments and the control studies also presented in Fig. 1 demonstrate that vigorous hydrolysis with acid was ineffective for cleavage of all the glucuronosides of the phenolic metabolites of estrone and estradiol. Furthermore, the amount recovered in the phenolic and neutral fractions by β-glucuronidase hydrolysis was appreciably greater than could be obtained by an additional 40 minutes refluxing with 5 per cent sulfuric acid. This again is excellent evidence that enzymatic cleavage is a far more efficient procedure for conjugates of estrogen metabolites than prolonged acid hydrolysis. That the radioactivity in the acidic fraction after acid hydrolysis was not present as sulfate esters is demonstrated by the control experiment in which the urine was extracted with ether at pH 1, a procedure known to be effective in the cleavage of sulfuric acid esters of steroids (18). Fig. 1 likewise gives evidence that secondary transformations of the estrogen metabolites occur on standing, since there was appreciable radioactivity in the aqueous fraction after exhaustive extraction with ether. Since this material had been obtained in an ether-soluble form prior to extraction with base in the previous separation, it is apparent that some alteration had occurred. These difficulties further emphasize the need for continued scrutiny of methodology to permit accurate conclusions about the urinary metabolites of natural estrogens.

Conversion to Estrone and Estriol—From the considerations dealt with earlier in the discussion, it seems clear that the methods employed in this investigation were adequate for obtaining a high but not necessarily quantitative yield of the two metabolites under discussion. As indicated in Table III, the recovered metabolites can be discussed in terms of either the dose administered, the total urinary radioactivity, or the metabolites found in the phenolic fraction. Of these three alternatives it would seem that the most realistic appraisal is in terms of the percentage of the urinary radioactivity. Consideration in terms of the dose administered alone af-

fords an unrealistically low estimate, because the estrogens are more slowly excreted than the metabolites of the neutral steroid hormones and even over several days only about 65 per cent of the radioactivity can be found in the urine. When expressed in terms of the phenolic fraction only, the values convey a misleading impression that a considerably greater quantity has been accounted for than there actually was.

From the data presented in Table III, it can be seen that the metabolite estriol was the major product in the urine of the 1st day after a tracer dose of either estrone or estradiol-17β. In the four experiments reported, an average value of 22 per cent of the urinary radioactivity was present as estriol, whereas the corresponding figure for estrone was 8.5 per cent. There seemed to be no relationship between the amount of either metabolite and the precursor steroid, whether estrone or estradiol-17β. While there were individual differences in the ratio of these two metabolites, in no instance was there more estrone than estriol. The relatively small recovery in Subject G is of some interest, but no explanation of the fact is apparent from the data.

TABLE III

Recovery of Radioactivity As Estrone and Estriol by Isotopic Dilution after Intravenous Administration of Estrone-16-C^{14} and Estradiol-17β-16-C^{14} in Women

Subject and compound administered	Estrone			Estriol		
	Per cent of			Per cent of		
	Phenolic	Urinary	Dose	Phenolic	Urinary	Dose
L. Estrone	19.8	10.2	4.8	28.5	14.8	6.9
R. "	11.9	7.7	2.6	47.5	30.9	10.3
G. Estradiol-17β	10.6	7.4	2.0	16.3	11.3	3.1
V. "	11.3	8.7	3.0	41.2	31.5	10.9

The importance of the manner of administration and the magnitude of the dose is emphasized by the results described in the following report (5). It is apparent, also, that the type and amount of metabolites must be investigated on successive intervals after the day of hormone administration. These and related problems will form the basis for further studies of estrogen metabolism in man.

SUMMARY

1. The excretion of radioactivity after administration of tracer doses of estrone-16-C^{14} and estradiol-17β-16-C^{14} was measured in six human females.

The urine contained the major portion of the metabolites; fecal excretion was minor in amount.

2. The pattern of radioactivity excretion was similar after either intramuscular or intravenous injection of estradiol-17β-16-C^{14}.

3. Use of β-glucuronidase was found superior to boiling with 5 per cent sulfuric acid for hydrolysis of the conjugated metabolites.

4. Estrone and estriol were measured by isotopic dilution in the urine of the 1st day after intravenous administration of estrone-16-C^{14} and estradiol-17β-16-C^{14} to four of the patients. Estrone accounted for from 8 to 10 per cent and estriol from 11 to 31 per cent of the total urinary radioactivity. The amount of these metabolites appeared to be similar for either precursor.

The authors express their gratitude to their colleague, Dr. Leon Hellman, for his cooperation in the studies with patients. We are especially indebted for their technical assistance to Mr. Albert Klutch, Mrs. Millicent Houde, and Mrs. Rosemarie Lehman. The radioactivity measurements in the gas phase were performed by Dr. Maxwell L. Eidinoff of this Institute, to whom we express our thanks for his assistance. Permission to administer radioactive isotope was obtained from the Atomic Energy Commission.

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EXCRETION OF ESTROGEN METABOLITES BY HUMANS*

II. THE FATE OF LARGE DOSES OF ESTRADIOL-17β AFTER INTRAMUSCULAR AND ORAL ADMINISTRATION

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The isolation and chemical characterization of metabolites of the estrogenic hormone estradiol-17β have been hampered by the paucity of these compounds in normal human urine as well as by uncertainty about adequate hydrolysis of the conjugates. While late pregnancy urine is a relatively rich source of estrone and estriol, there is little reason *a priori* to believe that these metabolites of pregnancy would exactly parallel the fate of tracer doses of hormone. Since our aim has been to develop the basic information requisite for a critical study of estrogen metabolism, it was necessary to obtain a sufficient quantity of metabolites to afford a reasonable opportunity for definite identification of any as yet unrecognized products of hormone metabolism. One of the means toward this end was the administration of large, admittedly unphysiologic, doses of estradiol-17β to human subjects in order to evaluate the feasibility of isolation procedures. At the same time it was desirable to have a comparison between the fate of large and small doses of the estrogen. The work described in this report was undertaken with this primary design.

In Paper I we have described certain initial investigations of the fate and metabolic transformation of tracer doses of estrogens with particular regard to intravenously administered hormone (1). Comparison of large and small doses given by this route is impractical because of the great insolubility of estradiol-17β in any vehicle suitable for injection into the vascular system. The hormone labeled with C^{14} was therefore injected intramuscularly into two subjects, and a comparison study was made in a third patient after a divided oral dose. After enzymatic hydrolysis by means of beef liver β-glucuronidase, the phenolic metabolites were isolated and measured by two methods, isotopic dilution and counter-current distribution, on each of 4 days after introduction of the hormone. Auxiliary studies of the hydrolysis of urinary conjugates, the excretion of radioactivity in urine and feces, and other details of estrogen metabolism were made.

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EXPERIMENTAL

Subject W (Female, 28 Years Old; Hodgkin's Disease)—Estradiol-17β-16-C^{14}, 10,500 c.p.m. per mg., was dissolved in sesame oil-benzyl alcohol, 3:1, so that each ml. contained 5.82 mg. of estradiol-17β-16-C^{14}. Single intramuscular injections, each of 3 ml., were made at eight consecutive 3 hour intervals. The total estradiol administered was 140 mg.; 1,464,000 c.p.m.

Subject H (Female, 53 Years Old; Carcinoma of Breast)—Estradiol-17β-16-C^{14}, 10,000 c.p.m. per mg., was dissolved in sesame oil-benzyl alcohol, 4:1, so that each ml. contained 6.055 mg. Eight consecutive intramuscular injections, each of 6.5 ml., were made at 3 hour intervals. The total estradiol administered was 315 mg.; 3,150,000 c.p.m.

Subject F (Female, 62 Years Old; Carcinoma of Breast)—A total of 350 mg. of estradiol-17β-16-C^{14}, 8850 c.p.m. per mg., was given orally in gelatin capsules in three equal doses at consecutive 12 hour intervals.

Collections—Urine collections were made for consecutive 24 hour periods; the start of the dose was the beginning of the first period. Feces were homogenized with water as soon as collected, and a known portion of the suspension was removed for isotopic analysis.

Fractionation—The ether extracts obtained after hydrolysis were separated into acidic, neutral, and phenolic components by the methods described in Paper I.

Estimation of Metabolites in Phenolic Fraction—Two alternative methods were used for measurement of the amounts of estrone, estradiol, and estriol in the urinary phenolic fractions: (1) isotopic dilution with known amounts of carrier compounds and (2) isolation and measurement of radioactivity of metabolites by counter-current distribution without added carrier.

Isotopic Dilution—This method was used with certain phenolic fractions of Subject W. Known weights of highly purified non-radioactive estrone, estradiol-17β, and estriol were added to known fractions of the phenolic extract. The latter was then separated into ketonic and non-ketonic fractions by Girard's Reagent T, and the products were isolated and purified.

Counter-Current Distribution—Known amounts of phenolic fractions were separated into ketonic and non-ketonic components by Girard's Reagent T. Each fraction was distributed in a 99 tube Craig counter-current machine. The solvent systems used were selected from those developed by Engel (2) and were as follows: *Ketonic fraction*, methanol 7, water 3, carbon tetrachloride 10 (parts by volume). The partition coefficient K was calculated from the formula of Williamson and Craig (3). The estrone peak was at tube 53 (K = 1.15). *Non-ketonic fraction*, equal volumes of ethyl acetate, cyclohexane, ethanol, and water. The estradiol peak was

at tube 68 ($K = 2.2$) and the estriol peak at tube 28 ($K = 0.4$). After distribution, the contents of each tube were withdrawn and the solvent removed. Known fractions of the residues were counted as solid mounts (accuracy ±10 per cent), and the observed radioactivity was plotted against the tube number to give the distribution curve of the metabolites. The observed curves were compared with the theoretical curves for the pure compounds calculated from the formula of Williamson and Craig (3).

When agreement between observed and theoretical distribution was good, the total amount of a compound present was calculated from the fraction found in the peak tube. This gives the following for the three

TABLE I

Excretion of Radioactivity after Large Doses of Estradiol-17β-16-C^{14}

Subject	W	H		F	
Route of administration	Intramuscular	Intramuscular		Oral	
Dose, *mg.*	140	315		350	
" *c.p.m.* × 10^{-3}	1464	3150		3098	
Day	Urine	Urine	Feces	Urine	Feces
	C.p.m. × 10^{-3}				
1st	178	627	0	553	5
2nd	422	715	122	840	303
3rd	231	385	*	390	250
4th	96	250	122	158	103
5th	16	141	27	39	62
6th	8	60	38	23	6
7th	3	43	43	12	12
8th	2.5	39		6	

* No feces excreted.

metabolites: estrone, peak tube 53 contains 8.05 per cent of the total estrone; estradiol, peak tube 68 contains 8.7 per cent of the total estradiol; estriol, peak tube 28 contains 8.95 per cent of the total estriol.

Isotopic Analysis—Radioactivity measurements were made by the methods previously described.

Excretion of Radioactivity—The daily urinary excretion of radioactivity by Subjects W, F, and H and the fecal radioactivity of Subjects F and H appear in Table I.

Distribution of Radioactivity in Urinary Fractions. (1) After Enzymatic Hydrolysis—The distribution of radioactivity after fractionation is shown in Tables II, III, and IV for Subjects W, H, and F, respectively.

(2) After Further Hydrolysis—The acidic and residual urine fractions of the 2nd day's enzyme-hydrolyzed urine from Subject W were combined,

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adjusted to pH 1 with sulfuric acid, and extracted for 48 hours with ether. The extract was then fractionated as usual. Relative to the combined acidic and residual urinary radioactivity (127,000 c.p.m.), the activity of

TABLE II

Radioactivity after Enzymatic Hydrolysis; Subject W, 1,464,000 C.p.m. Injected

	1st day	2nd day	3rd day	4th day
	C.p.m. × 10^{-3}			
Urine	178	422	231	96
Neutral	8	29	20	3.5
Acidic	12	15	20	6
Residual urine	71	184	55	34
Phenolic	97	213	112	48

Urine from the 5th day (total radioactivity, 16,000 c.p.m.) was treated with enzyme; 13,000 c.p.m. were found in the ether after 48 hours continuous extraction at pH 5.

TABLE III

Radioactivity after Enzymatic Hydrolysis and after Acid Hydrolysis of Combined Acidic Fraction with Residual Urine; Subject H, 3,150,000 C.p.m. Injected

	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day
	C.p.m. × 10^{-3}							
Urine	627	715	385	250	141	60	43	39
Enzyme								
Neutral	30	54	27	6	1	0.4		
Acidic	17	37	18	12	10	4		
Residual urine	171	268	168	116	58	26.5	15	11
Phenolic	420	386	166	129	67	33		
Acidic + residual urine refluxed with acid								
Neutral	4	9	4					
Acidic	60	46	34					
Residual urine	86	119	77	44	32	10		
Phenolic	44	115	70					

the fractions was as follows: acidic 39,000 c.p.m., neutral 1500 c.p.m., phenolic 11,000 c.p.m., and residual aqueous 72,000 c.p.m.

The combined acidic and residual aqueous fractions from the enzyme-treated urines of Subject H were refluxed for 4 hours with 5 per cent by volume of concentrated sulfuric acid. After cooling and extracting for 48

hours with ether, the metabolites were fractionated. The results appear in Table III. A sample of unhydrolyzed urine from the 1st day of Subject H (total urinary radioactivity, 627,000 c.p.m.) was adjusted to pH 1 with dilute sulfuric acid and extracted five times, each with 6 volumes of ether. The combined ether extracts were then fractionated in the usual manner. The distribution of radioactivity, calculated on the basis of the total urine, was as follows: acidic 368,000 c.p.m., neutral 9000 c.p.m., phenolic 61,000 c.p.m., and residual urine 200,000 c.p.m.

TABLE IV

Radioactivity after Enzymatic Hydrolysis and after Treatment of Residual Urine at pH 1 and with Boiling Acid; Subject F, 3,098,000 C.p.m. by Mouth

	1st day	2nd day	3rd day	4th day
	C.p.m. $\times 10^{-3}$			
Urine	553	840	390	158
Enzyme				
Neutral	28	46	19	9
Acidic	12	23	11	5
Phenolic	439	613	227	72
Residual urine extracted at pH 1				
Neutral	0.2	0.3	0.1	0.05
Acidic	15	16	22	10
Residual aqueous	37	75	100	55
Phenolic	2	4	3	2
Residual aqueous from extraction at pH 1, refluxed with acid				
Neutral	1	2	2	
Acidic	4	8	11	
Residual aqueous	22	38	22	
Phenolic	13	29	60	

The residual urine (Subject F) after enzyme hydrolysis was adjusted to pH 1 with sulfuric acid and extracted with ether for 48 hours. The radioactivity of the fractionated metabolites is reported in Table IV. The residual aqueous solution after ether extraction at pH 1 was then refluxed for 4 hours with 5 per cent by volume of concentrated sulfuric acid and reextracted with ether for 48 hours. The distribution of the metabolites after fractionation is shown in Table IV.

Radioactive Estrone, Estradiol-17β, and Estriol by Isotopic Dilution—The phenolic samples taken, the amount of carrier added, and the specific activities of the purified compounds were as follows:

Subject W. 1st Day—The total phenolic radioactivity was 97,000 c.p.m. To a portion of the phenolic fraction having 42,500 c.p.m. were added 50.0

mg. of estrone and 67.1 mg. of estradiol-17β. After isolation and purification the specific activities were as follows: estrone 139 c.p.m. per mg., estradiol-17β 45 c.p.m. per mg. To another portion of the phenolic fraction (38,500 c.p.m.) were added 47.3 mg. of estriol. The purified compound had a specific activity of 64 c.p.m. per mg.

2nd Day—The total phenolic radioactivity was 213,000 c.p.m. The phenolic sample taken for analysis had 39,000 c.p.m.; 49.6 mg. of estrone and 50.8 mg. of estriol were added. The specific activities of these compounds after isolation and purification were 235 c.p.m. per mg. for estrone and 62 c.p.m. per mg. for estriol. The value for estradiol was obtained by counter-current distribution (see below).

3rd Day—The total phenolic radioactivity was 112,000 c.p.m. The sample contained 48,000 c.p.m.; 46.9 mg. of estrone, 39.6 mg. of estradiol-17β, and 39.3 mg. of estriol were added as carriers. The extract was then treated with Girard's Reagent T as usual, and the two fractions were distributed in the counter-current machine. The three compounds thus isolated were collected and recrystallized to constant specific activity: estrone 149 c.p.m. per mg., estradiol-17β 72 c.p.m. per mg., estriol 176 c.p.m. per mg.

4th Day—The total phenolic radioactivity was 48,000 c.p.m. The phenolic sample taken for analysis had 39,000 c.p.m.; 61.1 mg. of estrone and 54.1 mg. of estriol were added, and, when isolated and purified, the specific activities were as follows: estrone 42 c.p.m. per mg., estriol 230 c.p.m. per mg.

Radioactive Estrone, Estradiol-17β, and Estriol by Counter-Current Distribution—The ketonic and non-ketonic fractions from known portions of the phenolic extract of enzyme-hydrolyzed urines were distributed in the Craig counter-current machine with the solvent systems described. The total amount of a particular compound was obtained by multiplying the peak tube radioactivity with the appropriate factor: estrone 100/8.05; estradiol 100/8.7; estriol 100/8.95. The radioactivity of the phenolic fractions used in the analyses and the radioactivity found in the peak tubes corresponding to estrone, estradiol, and estriol are given below.

Subject W. 2nd Day—The total phenolic radioactivity was 213,000 c.p.m. The phenolic sample taken for analysis contained 108,000 c.p.m.; estradiol peak tube, 810 c.p.m.

3rd Day—The total phenolic radioactivity was 112,000 c.p.m. The phenolic sample used for distribution in the machine had 46,000 c.p.m. The radioactivity in the peak tubes was 595 c.p.m. for estrone, 285 c.p.m. for estradiol, 710 c.p.m. for estriol.

Subject H. Phenolic Fractions from Enzyme-Hydrolyzed Urines. 1st Day—The total phenolic radioactivity was 420,000 c.p.m. The phenolic

sample taken for analysis had 388,000 c.p.m.; radioactivity in the peak tubes, estrone 6800 c.p.m., estradiol 5000 c.p.m., estriol 1700 c.p.m.

2nd Day—The total phenolic radioactivity was 386,000 c.p.m. The sample taken for analysis had 353,000 c.p.m.; peak tube radioactivities, estrone 4100 c.p.m., estradiol 1600 c.p.m., estriol 1400 c.p.m.

3rd Day—The total phenolic radioactivity was 166,000 c.p.m. The sample taken for analysis had 152,000 c.p.m.; the peak tube radioactivities, estrone 1500 c.p.m., estradiol 580 c.p.m., estriol 1300 c.p.m.

4th Day—The total phenolic radioactivity was 129,000 c.p.m. The sample taken for analysis had 116,000 c.p.m. The estrone and estradiol peaks were not well defined; the peak tubes had approximately 400 and 300 c.p.m. respectively; the estriol peak tube, 1800 c.p.m.

Subject H. Phenolic Fraction Isolated from Combined Acidic and Residual Urinary Fractions after Refluxing with Acid. 2nd Day—The phenolic extract used in this estimation was that obtained after refluxing with acid the combined acidic and residual urinary fractions of the 2nd day's enzyme-hydrolyzed urine. The total radioactivity of this phenolic fraction was 115,000 c.p.m. (Table III); the sample taken for distribution had 96,000 c.p.m. The estrone peak tube had 350 c.p.m., but the distribution curve was poorly defined. The estradiol and estriol peak tubes had 330 and 615 c.p.m. respectively. Calculated to the urinary phenolic radioactivity these gave 5000 c.p.m. for estrone, 5000 c.p.m. for estradiol, 8000 c.p.m. for estriol.

Subject F. Phenolic Fractions from Enzyme-Hydrolyzed Urines. 1st Day—The total phenolic radioactivity was 439,000 c.p.m. The sample taken for distribution had 410,000 c.p.m. The peak tubes corresponding to estrone, estradiol, and estriol had 18,500, 4700, and 360 c.p.m. respectively.

2nd Day—The total phenolic radioactivity was 613,000 c.p.m. The sample had 511,000 c.p.m. After distribution the activities in the peak tubes were 22,500 c.p.m. for estrone, 6600 c.p.m. for estradiol, 1900 c.p.m. for estriol.

3rd Day—The total phenolic radioactivity was 227,000 c.p.m. A sample with 210,000 c.p.m. was taken for distribution. The peak tube activities were 4500 c.p.m. for estrone, 1720 c.p.m. for estradiol, 1700 c.p.m. for estriol.

4th Day—The total phenolic radioactivity was 72,000 c.p.m. The sample taken for distribution had 66,000 c.p.m. The peak tube activities were 950 c.p.m. for estrone, 350 c.p.m. for estradiol, 1450 c.p.m. for estriol.

Subject F. Phenolic Fraction from Residual Aqueous Refluxed with Acid—The phenolic extract used in this distribution was that isolated from the 2nd day's residual aqueous after refluxing with sulfuric acid (Table IV);

total radioactivity, 29,000 c.p.m. The portion taken for distribution had 23,000 c.p.m., and the activities in the peak tubes were 95 c.p.m. for estrone, 60 c.p.m. for estradiol, 230 c.p.m. for estriol.

DISCUSSION

Excretion of Radioactivity—The urinary radioactivity following intramuscular administration of estradiol-17β corresponded to 65 per cent of the dose administered, and the major portion of this was eliminated in the first 4 days after introduction of the estrogen. The recovery was very similar for the two subjects and was almost the same as that found after the intravenous administration of a tracer dose (1). Indeed, if the 1st and 2nd days are considered together as the time of administration, the rate of elimination as well as the total recovery was very nearly identical for the small and the massive doses. It is apparent, therefore, that absorption from a large depot of oil was a remarkably efficient process and in gross aspect resembled intravenous introduction.

As with the small doses, the fecal excretion of radioactivity following a massive intramuscular dose was small in comparison with the amount given; *i.e.*, 11 per cent of the dose. The fecal metabolites, like those of urine, were present for several days and equaled the urinary excretion on the final day measured. In view of the suggestion that the molecule may undergo extensive degradation during a prolonged residence in the tissues of the recipient, it is perhaps significant that the proportion excreted through the gastrointestinal tract increased toward the end of the experimental period. A similar trend was seen after the oral dose when, on the 7th day, the fecal and urinary excretions of radioactivity were the same. The initially higher fecal excretion following the oral dose must, at least in part, be a consequence of incomplete absorption, although it is evident that a considerable fraction of the oral dose had been successfully absorbed. In fact, the radioactivity recovered in the oral experiment was almost 90 per cent of the amount given, and 65 per cent was in the urine.

Hydrolysis of Urinary Conjugates—It is apparent that only small amounts of unconjugated metabolites or easily hydrolyzed conjugates are present in urine. Thus, with Subject H, when the 1st day's urine was extracted with ether at pH 1 prior to any hydrolytic procedure, only about 10 per cent of the radioactivity in the urine appeared in the phenolic fraction. Comparison of the same day's urine after enzymatic hydrolysis, when 67 per cent of the urinary radioactivity was obtained in the phenolic fraction, strongly suggests that conjugation with glucuronic acid is the major means by which these metabolites are converted to water-soluble derivatives. This experiment likewise demonstrated that use of β-glucuronidase was an efficient

hydrolytic procedure for the conjugates of massive doses, as had already been shown for tracer quantities.

It is of some interest to note that in these experiments there was little evidence for the presence of sulfate esters of the metabolites of estradiol-17β. Thus after enzymatic hydrolysis of the 2nd day's urine of Subject W, when the combined acidic fraction and residual urine were continuously extracted with ether at pH 1, only 2.6 per cent of the urinary radioactivity was obtained in the form of phenolic metabolites. Similar results were obtained with Subject F after oral administration. After continuous extraction with ether at pH 1 of the urines from each of the first 4 days, the radioactivity of the phenolic fraction was negligible, ranging from 0.4 to 1.3 per cent of the radioactivity in the urine. The tentative conclusion seems warranted that glucuronosides are major and sulfate esters are very minor conjugates, although it is conceivable that the 5 day incubation at pH 5, in the presence of the enzyme, had hydrolyzed a portion of sulfate esters that were present.

The amount of radioactivity in the residual urine (Subject H, 1st day) after extraction at pH 1 without prior hydrolysis and after enzyme hydrolysis was almost the same, 32 and 27 per cent respectively. This can be interpreted as indicative of the existence of another type of conjugate, a markedly altered metabolite, or both. Further studies must be made in order to elucidate these problems, and these are at present in progress in our laboratories. It is evident, however, that glucuronosides and the metabolites in the "residual urine" comprise almost all the excretory products of estradiol-17β. For the most part, the conjugates are of phenolic compounds, since the neutral and acidic fractions are very small. When both the enzymatic and hot acid hydrolysates are considered together, about 70 per cent of the total metabolites in the urine are phenols.

The proportion of phenolic radioactivity after enzymatic hydrolysis was essentially similar on each of the days studied after intramuscular administration. There is more than a suggestion, however, that feeding the hormone alters both the pattern of conjugation as well as the nature of the metabolites produced. Whereas on the 1st and 2nd days of the oral experiment 79 and 73 per cent of the radioactivity were in the phenolic fraction after enzymatic hydrolysis, only 58 and 45 per cent were found in this fraction during the 3rd and 4th days. With this change there was a reciprocal increase in the residual urine radioactivity from the minimum of 10 per cent on the 1st day to 42 per cent on the 4th day. Since the principal metabolite during the first 2 days was estrone, this result with the oral dose is not very surprising, because this compound could form only a monoglucuronoside, readily cleaved by the enzyme. While it is evident that

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deduction from the categorical distribution of radioactivity is subject to considerable uncertainty, especially with respect to the presence or absence of enzyme inhibitors, it seems important to draw attention to these interpretations. It is recognized that the provisional conclusions must be verified by more detailed studies.

Finally it is to be noted that the radioactivity in the residual urine after enzyme hydrolysis contains some phenolic metabolites which can be cleaved from the conjugates by vigorous hydrolysis with acid. Thus on the 3rd day of Subject H, 18 per cent of the urinary radioactivity was obtained in the phenolic fraction after this treatment. A comparable amount in absolute terms, although smaller with respect to urinary radioactivity, was similarly converted to phenolic material in the first 2 days of the study.

It should be emphasized that the phenolic products recovered from the residual urine by prolonged boiling with acid contain very little of the three pure metabolites that have been investigated in this study. Thus of 115,000 c.p.m. in the phenolic fraction obtained after acid hydrolysis of the acidic plus residual urine of the 2nd day (Subject H), only 18,000 c.p.m. were accounted for by estrone, estradiol-17β, and estriol. With the exception of estriol, the additional amount of these metabolites obtained by this treatment would change the total recovery only slightly. Addition of the estriol from the acid hydrolysate would increase the total counts from 17,000 to 25,000; in terms of the dose this represents an increase from 0.55 to 0.81 per cent on that day. Even after combined enzymatic and acid hydrolysis, the residual aqueous solution still contained appreciable radioactivity.

Estrone, Estradiol, and Estriol—Of the two methods used for measurement of the three metabolites examined in this investigation, isotopic dilution is the more precise and unobjectionable on theoretical grounds. However, this is a laborious and time-consuming operation, ill suited to an extended study. While counter-current distribution has certain disadvantages about ease of operation, it affords information about other metabolites than the three compounds specifically investigated and many of the results reported were obtained by this procedure. A comparison of the results of the two methods was made with the 3rd day's urine of Subject W (see Table V). The agreement between the two procedures was such that, for the purpose of these studies, no serious differences would be encountered with the use of counter-current distribution alone. Care must be exercised, however, when the experimental curve departs appreciably from the theoretical distribution, and this has been noted wherever it occurred.

From the evidence presented in Table V it is manifest that estrone was the chief metabolite of the three compounds measured after a massive dose

of estradiol-17β either orally or intramuscularly. Estrone accounted for from 6 to 7 per cent of the dose, or 10 per cent of the total radioactivity in the urine, in the two intramuscular experiments during the first 4 days. Despite the fact that estradiol-17β was the compound administered, only from 2 to 3 per cent of the dose, or about 4 per cent of the total urinary

TABLE V

Radioactivity of Estrone, Estradiol, and Estriol after Intramuscular and Oral Estradiol-17β-16-C¹⁴

Subject, dose, and route of administration	Day	Phenolic	Estrone	Estradiol	Estriol
		C.p.m. × 10^{-3}			
W. 140 mg., 1,464,000 c.p.m., intramuscular	1st	97	16 (9.0)	7 (3.9)	8 (4.5)
	2nd	213	65 (15.4)	18 (4.3)	17 (4.0)
	3rd	112	17 (7.4)	7 (3.0)	16 (6.9)
	4th	48	3 (3.1)		16 (16.7)
H. 315 mg., 3,150,000 c.p.m., intramuscular	1st	420	91 (14.5)	62 (9.9)	21 (3.3)
	2nd	386	56 (7.8)	20 (2.8)	17 (2.4)
	3rd	166	20 (5.2)	7 (1.8)	16 (4.2)
	4th	129	*Ca.* 6 (2.4)	*Ca.* 4 (1.6)	22 (8.8)
F. 350 mg., 3,098,000 c.p.m., oral	1st	439	246 (44.5)	58 (10.5)	4 (0.7)
	2nd	613	335 (39.9)	91 (10.8)	25 (3.0)
	3rd	227	60 (15.4)	21 (5.4)	21 (5.4)
	4th	72	13 (8.2)	4 (2.5)	18 (11.4)

The figures in parentheses represent the per cent of the total radioactivity excreted in the urine on that day. The values reported for Subject W were obtained by isotopic dilution, except for the estradiol value for the 2nd day. The values for Subjects H and F were obtained by counter-current distribution. The phenolic fraction, 3rd day of Subject W, was measured by both methods. The values in counts per minute × 10^{-3} obtained by counter-current distribution were 18 for estrone, 8 for estradiol, 19 for estriol. The combined acidic and residual urine fractions after enzymatic hydrolysis of the urine from Subject H, 2nd day, contained, in counts per minute × 10^{-3}, 115 for the phenolic fraction, 5 for estrone, 5 for estradiol, 8 for estriol. The phenolic fraction (29,000 c.p.m.), Subject F, 2nd day, from the residual aqueous solution after refluxing with sulfuric acid, contained, in counts per minute × 10^{-3}, 2 for estrone, 1 for estradiol, 3 for estriol.

radioactivity during the first 4 days, was recovered as this compound after intramuscular injection. The amount of estriol was intermediate between these two substances, from 2 to 4 per cent of the dose administered, or about 5 per cent of the total radioactivity in the urine during the first 4 days after intramuscular injection.

As time after administration progressed, the amount of estrone and estradiol diminished rapidly, and by the 4th day relatively minor amounts of these metabolites were found in urine. Estriol, on the other hand, was

excreted at a relatively constant rate when expressed in terms of per cent of the dose, but at a rapidly increasing rate if considered in terms of the urinary radioactivity. Estriol on the 4th day of Subject W corresponded to 17 per cent of the radioactivity in the urine, compared to 3.1 per cent for estrone. In Subject H, a similar but not quite so marked difference was evident, in that estriol represented approximately 9 per cent of the urinary radioactivity on the 4th day, compared to 2.4 per cent for estrone.

After estradiol given orally, estriol was an insignificant metabolite on the 1st day, but by the 4th day had increased to 11.4 per cent of the total radioactivity in the urine. Estrone accounted for 45 per cent of the urinary radioactivity on the 1st day, but had diminished to 8 per cent of the urinary radioactivity by the 4th day. The excretion of estradiol-17β was significantly greater than that of estriol and over the 4 days studied accounted for 5.6 per cent of the dose, most of which was obtained during the first 2 days. It is to be noted that the recovery of radioactivity as these three estrogens was considerably higher in the oral experiment, 29 per cent of the dose, than in the intramuscular experiments, 11 and 13 per cent of the dose. It would appear that most of the oral dose was quickly conjugated in either the intestinal mucosa or the liver and excreted after only minor chemical transformation. A small portion of the dose, however, appeared to undergo the same type of metabolic alteration found after intramuscular administration, in that it was retained for a relatively long time and was converted in part to estriol as well as to the metabolites found in the residual urine. Since estradiol is comparatively inactive orally, the fact that a considerable portion was excreted as estrone, a biologically active metabolite, is a strong indication that conjugation had transformed this product into biologically inert material.

The most marked contrast between the small and the massive doses of estradiol-17β is found in the relative proportion of the three metabolites. With the small doses, estriol was the principal metabolite on the 1st day and was excreted in larger amount than estrone in all the subjects studied. After the large dose either intramuscularly or orally the reverse was true in that excretion of estrone was significantly higher than that of estriol. The fact that both oral and intramuscular doses were chiefly converted to estrone is suggestive that the size of the dose rather than the manner of introduction is the determinant in the metabolic transformation. While an unobjectionable conclusion is precluded because of the several variables as well as the small group of patients studied, the significance of the result is considerable. It is quite possible that, had the dynamics of the small dose been as completely investigated as of the massive amount, even more striking differences might have been disclosed. A further study of these differences in ratio and amount of metabolites is of manifest interest

for the interpretation of the estrogen excretion in human pregnancy. It may be suggested from these results that the relatively large production of hormones in late pregnancy leads to an altered metabolic course which may in turn materially influence the rôle of these substances in the maternal tissues. Further investigation of these problems is clearly necessary.

It would be desirable to compare the results obtained in these studies of large doses with the recent report of Pearlman, Pearlman, and Rakoff (4) who administered large doses of estrone acetate to pregnant women. While these investigators used a deuterium-labeled estrone, their results, unfortunately, were based upon bioassay of a crude fraction rather than upon the isotope. Although an accuracy of ±20 per cent is claimed for the bioassay, it is clear from the results recorded that very much greater errors were encountered. For these and other reasons, including the hydrolytic procedure used by these authors, comparison of their results with the present study is impossible.

SUMMARY

1. After intramuscular and oral administration of large doses of estradiol-17β-16-C^{14} to women, the dynamics of metabolism and excretion were studied at daily intervals until the radioactivity had diminished to very low levels. Approximately 65 per cent of the radioactivity injected was found in the urine; the fecal radioactivity was small in amount.

2. Further investigations of the hydrolysis of urinary conjugates were made and compared with the results obtained after tracer amounts of hormone.

3. Measurement of estrone, estradiol-17β, and estriol in the urine from each of the first 4 days after the dose was achieved by two methods, isotopic dilution and counter-current distribution. Estrone was the principal metabolite during and immediately after either injection or feeding. As time after the dose progressed, estriol became the major metabolite isolated from the urine. The amount of estradiol-17β was less than that of estrone and greater than that of estriol.

4. The results were compared with those found after a tracer dose, and the provisional conclusion was drawn that the difference was a consequence of the size of the dose rather than of the other variables considered.

The authors express their gratitude to their colleague, Dr. Leon Hellman, for his cooperation in the studies with patients. We are especially indebted for their technical assistance to Mr. Albert Klutch, Mrs. Millicent Houde, and Mrs. Rosemarie Lehman. The radioactivity measurements in the gas phase were performed by Dr. Maxwell L. Eidinoff of this Insti-

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tute, to whom we express our thanks for his assistance. Permission to administer radioactive isotope was obtained from the Atomic Energy Commission.

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THE ISOLATION AND CHARACTERIZATION OF DERMATITIC COMPOUNDS PRODUCED BY MYROTHECIUM VERRUCARIA

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A number of reports on dermatitic or skin-irritating compounds produced by various molds have appeared in the recent literature. Several Russian workers, Vertinsky (1), Moseliani (2), Salikov (3), and Drobotko (4), have described the poisonous effects of the mold *Stachybotrys alternans* Borod. which produces a disease in horses known as stachybotryotoxicosis. The disease is caused by the consumption of hay on which the mold has been growing and is characterized by severe lesions of the nose, mouth, throat, and gastrointestinal tract. Ether extracts of cultures of the mold have dermatitic properties. Brian, Curtis, and Hemming (5) while handling large quantities of culture fluid during the isolation of glutinosin, an antifungal agent produced by *Metarrhizium glutinosum* S. Pope, experienced severe facial inflammation which was attributed to a volatile dermatitic compound produced by the organism. Lesions were also produced by dipping small filter paper disks in the culture fluid and strapping them in contact with the skin of the forearm for 48 hours. In later studies Brian, Hemming, and Jeffreys (6) found that other strains of the same fungus and several strains of *Myrothecium roridum* also produced dermatitic compounds. The present report deals with the production of several dermatitic compounds produced by *Myrothecium verrucaria* and the isolation and characterization of one of these compounds. The organism *M. verrucaria* (Alb. and Schw.) Ditm. ex Fr. used in these experiments has been shown by White and Downing (7) to be identical with the species *Metarrhizium glutinosum* S. Pope.

EXPERIMENTAL

Culture Medium—The medium used for the production of dermatitic compounds was a modification of Medium AS described by Brian, Curtis, and Hemming (5) and consisted of 50 gm. of glucose, 1.0 gm. of KH_2PO_4, 0.5 gm. of $MgSO_4 \cdot 7H_2O$, 1.0 gm. of $(NH_4)_2SO_4$, 1 mg. of $FeSO_4 \cdot 7H_2O$, 0.15 mg. of $CuSO_4 \cdot 5H_2O$, 1.0 mg. of $ZnSO_4 \cdot 7H_2O$, 0.1 mg. of $MnSO_4 \cdot 7H_2O$, and 0.1 mg. of K_2MoO_4 per liter of distilled water. The starting pH of all cultures was adjusted to 4.0.

Aeration and the addition of certain organic acids to the culture medium

were found to increase the yield of dermatitic compounds considerably (Table I). Of the organic acids tested the most effective were malic and tartaric. The best medium used for the production of the dermatitic compounds in quantities suitable for isolation, therefore, contained in addition to the ingredients mentioned in the preceding paragraph 1 per cent tartaric acid and 0.2 per cent malic acid.

Production of Dermatitic Compounds—For large scale production of the dermatitic compounds, 12 liter batches of medium were prepared and placed in 20 liter glass carboys. A typical isolation experiment was carried out in the following manner. Two 12 liter batches of medium were placed in individual glass carboys. The inoculum was prepared by suspending the spores from a surface culture of the mold (grown on Czapek-

TABLE I

Effect of Added Organic Acids on Sugar Utilization, pH, and Dermatitic Compound Production of Cultures of M. verrucaria

Acid added (1 per cent)	Glucose utilized	Final pH	Dermatitic compound
	per cent		*mg. per l.*
None	16	2.5	20
Tartaric*		3.8	8
Tartaric	76	4.3	94
Malic	70	5.0	58
Malonic	96	5.2	43
Oxalic	79	7.4	22

* This series was not agitated during incubation.

Dox agar in an 8 ounce glass bottle) in 5 ml. of autoclaved distilled water. 1 ml. of the spore suspension was placed in 100 ml. of medium in a 250 ml. Erlenmeyer flask. The flasks were placed in a rotating shaker which operated at 100 cycles per minute, and the culture was allowed to grow for 7 days at 25°. The medium in each carboy was inoculated with 100 ml. of this 7 day-old culture. The large cultures were also incubated at 25° and were aerated constantly during the incubation period by blowing filtered air into the cultures through sintered glass disks. Periodic analyses were made on portions of the medium during the growth period to follow the progress of dermatitic compound production.

Analytical Methods—Analytical procedures used for the determination of these compounds were based on biological activity, ability to absorb ultraviolet light, and chemical properties. The biological assay was carried out by allowing 0.1 ml. of alcoholic solution to dry on a rabbit's skin from which the hair had been removed with a pair of small animal clippers. The greatest dilution which would just give a visible lesion in 48 hours was

taken as a measure of the quantity of dermatitic substance in the preparation. Later studies showed that the minimal quantity of purified active compound detected by the bioassay was 0.3 γ. A more convenient method was found for determining the concentration of the dermatitic substances which was simply to measure the ultraviolet light absorption of a diluted aliquot of the culture at 263 mμ. A third procedure consisted of determining the light absorption at 450 mμ of the reaction product formed when diazobenzenesulfonic acid was added to a solution of the active compounds. This method was adapted from a procedure by Snell and Snell (8) and was carried out by adding 1 ml. of the reagent to a properly diluted aliquot of the culture in 1 per cent sodium carbonate solution and measuring the absorption after 2 minutes. The optical methods, though not specific for the determination of the dermatitic compounds, were found to be sufficiently selective, when based on the bioassy, to be used as a rapid means for determining the concentration of the dermatitic compounds during the production and isolation.

Isolation of Biologically Active Compounds—At the end of 4 weeks when the concentration of the active compounds in the carboy cultures had reached a maximum, the cultures were filtered and the combined filtrates extracted three times with 2.4 liters of ethyl ether. Care was taken during this operation to protect the face against burns from volatile dermatitic substances by wearing a gas mask. All exposed skin surfaces were covered with a barrier cream. The product known as Chemiglov proved satisfactory. The ether extract was evaporated under a vacuum and the brown gummy residue extracted with 240 ml. of benzene. Benzene was found to be a more selective solvent for the dermatitic compounds than ethyl ether, but was not used in the initial extraction because of its tendency to form emulsions. The benzene was evaporated *in vacuo* and the residue dissolved in 5 ml. of butyl alcohol. This solution was prepared for a 100 transfer counter-current distribution in the Craig machine. The solvent system used in the Craig machine was composed of 4850 ml. of ligroin (b.p. 100–110°), 150 ml. of *n*-butanol, and 5000 ml. of 0.1 M acetate buffer, pH 4.0. When the components of this system are mixed, two layers of nearly equal volume are produced. A 100 glass tube Craig machine was used, each tube containing 40 ml. of each phase of the solvent system. Because of the relatively low solubility of the dermatitic compounds in the solvent system it was necessary to add the butanol concentrate to 395 ml. of the system (adjusted to allow for the extra butanol added) which in turn was divided into five equal portions and added to tubes 0 through 4 of the Craig machine. Both phases of the solvent system were added to the remainder of the tubes and the counter-current distribution was carried out in the usual manner at room temperature.

At the end of the Craig extraction the amount of dermatitic compound in each of the tubes was determined by the diazobenzenesulfonic acid method. The distribution of these compounds is presented in Fig. 1. The contents of tubes 35 to 50 were chosen for further purification and characterization studies, since the distribution of the substance reacting with the diazo reagent in these tubes closely resembled the theoretical distribution of a single substance. The distribution curves of the compounds reacting with the diazo reagent in tubes 0 to 20 and 70 to 99, however, were too wide to fit the calculated distribution curves of single substances;

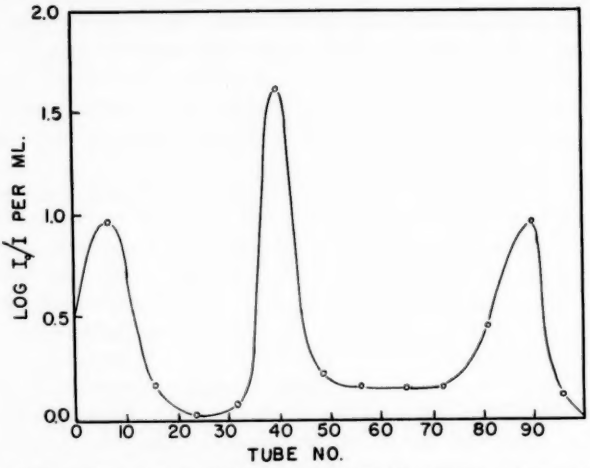

FIG. 1. Distribution of dermatitic compounds in the Craig machine. Absorption measurements were made at 450 mμ of the colored compound formed on addition of diazobenzenesulfonic acid.

hence further resolution of these mixtures would be necessary before characterization studies could be carried out on the pure compounds. High dermatitic activity was found only in tubes 35 to 50 and 70 to 99.

The dermatitic compounds present in other cultures of *M. verrucaria* when subjected to the fractionation procedure described above were found to produce Craig distribution patterns which varied to some extent from the one given in Fig. 1, but in all cases two or more compounds were detected. The partition coefficients of each of these compounds, however, seemed to fall close to one of the following values: 0.1, 0.9, 4.0, and 11.0.

In the typical experiment described above, the contents of tubes 35 to 50 were combined, the solvents were removed by vacuum distillation, and the remaining material was extracted three times with 200 ml. of ether.

The ether extracts were combined and the solvent was removed by evaporation *in vacuo*. The residue, an oily liquid, weighed 280 mg. When this residue was lyophilized from 20 ml. of benzene, a white microcrystalline residue was produced.

Characterization of Crystalline Compound—The crystals melted at 38°. An alcoholic solution of the compound had a specific rotation of $[\alpha]_D^{25}$ +94°. The compound consisted solely of carbon, hydrogen, and oxygen. No nitrogen, sulfur, phosphorus, or halogen was found on analysis, and no ash was produced on combustion. The molecular weight as determined by freezing point depression of a benzene solution was 270 ± 15. The molecular formula $C_{15}H_{22}O_4$ fits best the molecular weight determination and the analyses.

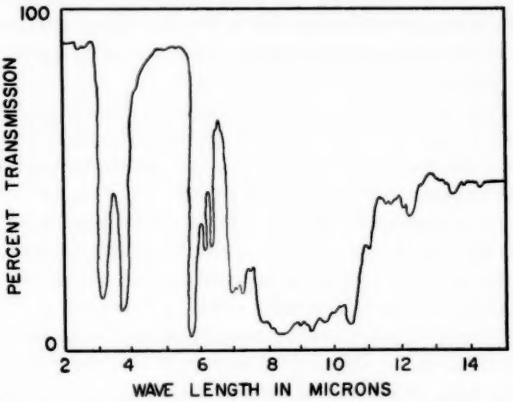

FIG. 2. Infra-red spectrum of the dermatitic compound isolated from tubes 35 through 50 of the Craig machine.

$C_{15}H_{22}O_4$ (266.3). Calculated, C 67.6, H 8.3; found, C 68.1, H 8.4

Analysis for methoxy groups revealed that none was present. Methylation of the compound was accomplished in the following manner. 5 ml. of a 2 per cent ether solution of diazomethane were added to 8.9 mg. of the compound in 1 ml. of ethyl alcohol. The solution was stored at 5° for 24 hours after which the solvents were evaporated and the sample was reweighed. A gain in weight of 1.24 mg. was noted which is equivalent to the addition of about three methyl groups.

The dermatitic compound was very soluble in chloroform, benzene, ethanol, methanol, butanol, and ether, but considerably less soluble in water, ligroin, and carbon tetrachloride. The compound was weakly acidic, since

it could be extracted into aqueous solutions at pH 11 but not at pH 8. Decomposition as observed by loss of dermatitic activity and discoloration took place rapidly above pH 11. The compound reacted readily with diazobenzenesulfonic acid to give a yellow color. It also produced a bright red color in the Liebermann nitrous acid reaction (9) but failed to form a colored complex with ferric chloride in methanol in concentrations as high as 1 mg. per ml. Millon's test ((9) p. 134) and the titanous chloride test of Weygand and Csendes (10) were negative.

An aqueous solution (pH 6.5) of the compound absorbed in the ultraviolet with a single maximum at 263 mμ (ϵ = 7740). A dried film of the compound was prepared on a silver chloride plate for infra-red absorption studies which were carried out with a Perkin-Elmer spectrophotometer model 12C. Major absorption peaks occurred at the following wavelengths: 3.0, 3.5, 5.85, 10.4, and 12.3 μ (Fig. 2).

Similar Compounds Present in M. verrucaria Cultures—Another compound isolated from a different batch of culture filtrate had a partition coefficient of 0.12 in the liquid counter-current distribution system. Upon fractionation the compound was distributed in the Craig machine relatively free of other overlapping substances. It was isolated as a yellow oil, and the yield per liter of culture was 1.4 mg. The compound had very little dermatitic activity and an alcoholic solution had no optical rotation. Like the first compound it contained only carbon, hydrogen, and oxygen, but the carbon content, 58.1 per cent, was appreciably lower than that of the more active compound. The molecular weight was determined as 253 $\pm$ 10. The ultraviolet and infra-red spectra of the two compounds were very similar.

At least two more compounds were detected in *M. verrucaria* culture filtrates which have distribution coefficients in the two phase solvent system near 4 and 10. These compounds have not been isolated, although mixtures of crystals have been obtained. Preliminary studies have shown that these compounds have higher molecular weights and greater optical rotation than the compounds previously described. They also possess high dermatitic activity.

DISCUSSION

According to the analytical data presented for the first crystalline dermatitic compound described, a likely explanation for the weakly acidic properties is the presence of a phenolic structure. The hydroxyl group is strongly indicated by the infra-red absorption at 3.0 μ. NH structures which also show a maximum in this region can be eliminated because of the absence of nitrogen in the compound. The maximum at 3.5 μ can most probably be attributed to CH structures, while the peak at 5.85 μ is most

often associated with the C=O double bond. In view of the weakly acidic properties of the compound it seems more likely that this structure can be attributed to an aldehyde, ketone, or ester group than to a carboxyl group. Ester groups are probably somewhat less likely to be present than aldehyde or ketone groups, since the ester maximum in this region usually falls closer to 5.75 than 5.85 μ. In general the infra-red spectrum, including the maxima at 10.4 and 12.3 μ, is quite characteristic of aromatic or alkene structures. The aromatic nature of the compound is confirmed by the positive Liebermann reaction and the production of a yellow color with diazotized sulfanilic acid. If the compound is phenolic in nature the absence of color production in the presence of ferric chloride indicates polysubstitution on the aromatic ring. The positive diazotized sulfanilic acid and Liebermann reactions indicate an unoccupied para position, while the negative Millon's test is characteristic of di-*o*- and di-*m*-substituted phenols. The negative titanous chloride test reveals the lack of *o*-quinone structures. In spite of the evidence for an aromatic ring, the ratio of hydrogen to carbon atoms in the molecular formula $C_{15}H_{22}O_4$ is too great to permit a completely aromatic structure to be written for the compound; thus a partially aliphatic structure must be considered.

The skin-irritating compounds of poison ivy, which possess aromatic rings, hydroxyl groups, aliphatic side chains, as well as dermatitic activity, also represent an interesting series. These and other natural products of similar chemical and biological properties have been reviewed by Wasserman and Dawson (11).

SUMMARY

At least three compounds possessing high dermatitic activity have been produced by culturing the organism *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr. One of these compounds has been isolated in crystalline form and its molecular formula determined as $C_{15}H_{22}O_4$. Another compound produced by the mold and isolated as a yellowish oil had low biological activity but was chemically and spectrophotometrically similar to the active compounds.

The authors are indebted to Dr. Edwin V. Hill and to Dr. James D. Mold for suggestions on the problem, to Dr. P. W. Brian of Butterwick Laboratories, Welwyn, Herts, England, for supplying the organism, to Dr. Benjamin Warshowsky of Camp Detrick and Mr. Erna J. H. Rhodes of the Army Chemical Center for analyses, and to Dr. George S. Melville and Mr. Edward J. Weneck of Camp Detrick for infra-red spectrophotometric determinations.

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THE CONVERSION OF FRUCTOSE-1-C^{14} AND SORBITOL-1-C^{14} TO LIVER AND MUSCLE GLYCOGEN IN THE RAT*

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It has been previously demonstrated (1, 2) that the metabolism of fructose in rat liver extracts involves the cleavage of fructose-1-phosphate to two 3-carbon fragments. If the same mechanism operates *in vivo*, the glycogen formed in the liver after administration of fructose-1-C^{14} to the intact rat should have C^{14} mainly in C-1 and C-6 of the glucose units. A similar distribution of C^{14} in liver glycogen can be expected after administration of sorbitol-1-C^{14}. Blakley (3) has shown that this polyalcohol is oxidized to fructose in liver extracts by a corresponding dehydrogenase; indirect evidence also indicates that in the intact animal fructose rather than glucose is formed from sorbitol. The paper by Stetten and Stetten (4) gives a more complete discussion of this problem.

On the other hand, Cook and Lorber (5) have shown that glycogen formed in liver and muscle after injection of glucose-1-C^{14} contains the major proportion of the radioactivity in C-1, the rest being more or less uniformly distributed among the other 5 carbon atoms.

In the present report, experiments are described in which 1-C^{14}-labeled fructose, glucose, and sorbitol were injected into rats and the isotope distribution patterns of the liver and muscle glycogen were determined.

Since direct utilization of fructose by muscle is known to be slow (6), it is uncertain whether the labeled glycogen formed by this tissue when C^{14}-fructose is given to the rat arises directly from the fructose or whether it is derived from radioactive glucose newly formed from fructose in the liver. This conversion complicates the interpretation of data obtained with muscle. In order to obviate this difficulty, experiments have been carried out with isolated rat diaphragm. The uptake of fructose by this preparation was previously shown by Haft *et al.* (7) and by Mackler and Guest (8) to be increased by the addition of insulin.

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Methods

Materials—Fructose-1-C^{14} and glucose-1-C^{14} were prepared by a method previously described (9). Sorbitol-1-C^{14} was obtained by reducing 50 mg. of glucose-1-C^{14} with Raney nickel according to Karabinos and Ballun (10). The yield of sorbitol was small (less than 20 per cent of the theoretical yield) because most of it could not be eluted from the catalyst. The sorbitol-1-C^{14} was separated from a small amount of radioactive contaminant by chromatography on a sheet of Whatman No. 1 filter paper (57 × 47 cm.) with water-saturated phenol (11) as solvent. The labeled sorbitol was located by exposing the dry sheet to x-ray film and was then eluted with water. By two-dimensional paper chromatographic analysis in water-saturated phenol and in butanol-acetic acid-water solvents (11), the product was shown to be free from any radioactive contaminants. Uniformly labeled fructose, prepared photosynthetically (12), was contributed by Dr. E. W. Putman.

Experiments with Intact Rats—Adult male rats of the Long-Evans strain, weighing between 200 and 270 gm., were used. After fasting for 24 hours, these animals received 1.5 ml. of a 50 per cent glucose solution by stomach tube. 20 minutes later they were given an intracardiac injection[1] of 20 mg. of the radioactive compound as a 5 per cent solution. The animals were killed by decapitation 1 or 3 hours after the injection. The liver and the carcass were digested separately in hot 30 per cent KOH and the glycogen was precipitated by addition of 1.2 volumes of 95 per cent ethanol. The crude glycogen was dissolved in hot water, separated by centrifugation of any insoluble material, and reprecipitated. This procedure was repeated again. The final glycogen solution was dialyzed against distilled water overnight and hydrolyzed with 0.06 N H_2SO_4 at 100° for 3 hours. The acidic solution was neutralized with barium hydroxide and separated from the barium sulfate precipitate by centrifugation. It was finally treated with charcoal and passed over a column containing a mixture of anion and cation exchange resins (Duolite A-4 and Amberlite IR-120).

Before dialysis an aliquot of the glycogen solution was mounted on an aluminum plate and its radioactivity determined with a thin end window Geiger tube.

Experiments with Isolated Rat Diaphragm—Normally fed rats were killed by decapitation and the diaphragm was excised and immersed for 10 minutes in ice-cold Krebs-Henseleit bicarbonate buffer (13) previously saturated with 95 per cent oxygen-5 per cent carbon dioxide. From two to four pieces were obtained from each hemidiaphragm. Each piece was blotted dry, weighed, and placed in the center well of an Erlenmeyer flask having a double compartment (14). The incubation was done in a small

[1] Kindly performed by Dr. R. Hill of the Department of Physiology.

volume (0.25 or 0.5 ml.) of the Krebs-Henseleit bicarbonate buffer containing 200 mg. per cent of radioactive fructose. When insulin was used, 1 unit of amorphous Novo insulin was added. The flasks were aerated with 95 per cent oxygen-5 per cent carbon dioxide, closed with a rubber cap, and shaken for 2 hours in a water bath at 37°. 0.5 ml. of 1 N acetic acid was then injected into the center well through the rubber cap and 1 ml. of 1 N NaOH into the main compartment of the flask. After allowing half an hour for CO_2 absorption, the diaphragm pieces were blotted, washed, and digested with 0.5 ml. of hot 30 per cent KOH. 0.2 ml. of 10 per cent Na_2SO_4 was added and the glycogen was precipitated in 60 per cent ethanol, isolated by centrifugation, and redissolved in 1 ml. of water. This procedure was repeated twice. An aliquot of the solution was evaporated on an aluminum plate and assayed for radioactivity. The total amount of CO_2 was determined by back-titration of the NaOH in the presence of $BaCl_2$. The specific activity of the resulting barium carbonate was determined by plating the salt on aluminum disks and assaying as before and correcting for mass absorption.

Degradation of Glucose—Glucose was degraded by fermentation with *Leuconostoc mesenteroides* according to a modification of the procedure of Gunsalus and Gibbs (15). In this fermentation, glucose yields quantitatively CO_2 from carbon atom 1, ethanol from carbon atoms 2 and 3, and lactate from carbon atoms 4, 5, and 6.

80 mg. of glucose were incubated at 30° with the amount of washed cells required to complete the fermentation in about 40 minutes. The incubation was done under reduced pressure in order to favor the diffusion of the CO_2 to the center well containing the NaOH. After 1 hour the cells were removed from the incubation mixture by high speed centrifugation. The ethanol was recovered by distillation and oxidized to acetate by heating for 10 minutes on a steam bath in 8 N sulfuric acid in the presence of 100 mg. of potassium dichromate. The non-volatile fraction was concentrated to about 0.5 ml. and mixed with 20 times its weight of anhydrous $KHSO_4$. The resultant dry salt was packed in a glass tube and the lactic acid was extracted by 50 ml. of ether. Lactate and acetate were degraded according to Katz, Abraham, and Chaikoff (16). For total combustion of glucose, alcohol, or acetate, potassium persulfate was used according to Katz, Abraham, and Baker (17). Each of the 6 carbon atoms, as well as carbon atoms 2 and 3 together (alcohol) and 5 and 6 together (acetate), was converted to barium carbonate and C^{14} was estimated.

Results

Experiments with Intact Animals—Data on the recovery of C^{14} in the glycogen of rat liver and muscle after administration of C^{14}-labeled sugars and sorbitol are presented in Table I. The distribution patterns of C^{14} in

glycogen formed by the rat from various precursors are given in Table II. Both liver and muscle glycogen formed from fructose-1-C^{14} contained most of the radioactivity in the terminal carbon atoms, with about twice as

TABLE I

C^{14} Recovery in Glycogen after Administration of Labeled Carbohydrates to Rats

Experiment No.	Compound injected	Amount of C^{14} injected	Duration of experiment	Rat body weight	Per cent of injected C^{14} recovered in	
					Liver glycogen	Muscle glycogen
		c.p.m.	*hrs.*	*gm.*	*per cent*	*per cent*
1	Fructose-1-C^{14}	1.3×10^6	1	200	18.0	5.6
2	"	1.7×10^6	3	220	25.0	7.5
3	Glucose-1-C^{14}	1.7×10^6	1	200	12.2	5.1
5	Sorbitol-1-C^{14}	2.5×10^5	1	220	13.0	4.0
6	"	2.5×10^5	3	270	14.6	

TABLE II

Distribution of Labeling in Liver and Muscle Glycogens in Rats 1 and 3 Hours after Injection of 1-C^{14}-Labeled Fructose, Glucose, and Sorbitol

The results are expressed in per cent of the total radioactivity of the glycogen.

Carbon atom	Fructose-1-C^{14}				Glucose-1-C^{14}				Sorbitol-1-C^{14}		
	1 hr.		3 hrs.		1 hr.		3 hrs.		1 hr.		3 hrs.
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver
1	54	65	51	56	86	95.0	65	84	60	57	46
2	5	2	6	3	4	0.9	7	2	2 *	2 *	4 *
3	2	1	2	1	2	0.3	4	1	2	2	4
4	2	3	3	1	1	0.4	6	1	1	2	2
5	4	2	4	2	3	0.4	7	2	3	1	4
6	30	25	29	26	4	0.5	7	2	32	33	26
Recovery....	97	98	95	89	100	97.5	96	92	100	97	86

* The brace indicates that the 2 carbons have been counted together after persulfate oxidation of the alcohol.

much in C-1 as in C-6. The labeling patterns of glycogen are virtually the same in the 1 and 3 hour experiments. Control experiments showed that most of the radioactivity in glycogen derived from glucose-1-C^{14} was in C-1, the remainder being distributed among the other 5 carbons without special localization in C-6. The extent of randomization increased with the duration of the experiment.

The injection of sorbitol-1-C^{14} resulted in approximately the same distribution of C^{14} as was obtained with fructose-1-C^{14}.

TABLE III

Recovery of C^{14} in Glycogen and CO_2 Formed from Uniformly C^{14}-Labeled Fructose by Isolated Rat Diaphragm

0.5 mg. of uniformly labeled fructose, containing 100,000 c.p.m., is present in 0.25 ml. of the incubation medium. The data are expressed for 50 mg. of tissue.

Glucose added	Insulin	Experiment 1		Experiment 2		Experiment 3	Experiment 4
		Glycogen	CO_2	Glycogen	CO_2	Glycogen	Glycogen
		c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*
None	−	2260	1520	830	1970	1140	1310
"	+	4100	2680	1380		2300	2820
0.2%	−	1075				380	
0.2%	+	1630					
2%	−	109	370	39	460	10	
2%	+	85	370	36			

TABLE IV

Distribution of C^{14} in Glycogen Formed from Fructose-1-C^{14} in Isolated Rat Diaphragm

Two hemidiaphragms were incubated separately for 2 hours at 37°, without and with insulin, in 0.5 ml. of Krebs-Henseleit solution containing 1 mg. of fructose-1-C^{14} (10^6 c.p.m.). When no insulin was added, 30,000 c.p.m. were recovered in the glycogen; with insulin, 60,000 c.p.m. were recovered. The results are expressed in per cent of the total radioactivity of the glycogen.

Carbon atom	Without insulin	With insulin
1	90.5	94.0
2	0.5	0.5
3	0.5	0.5
4	0.3	0.7
5	0.7	0.3
6	6.0	5.0
Recovery	98.5	101.0

Experiments with Isolated Rat Diaphragm—Table III shows that the synthesis of glycogen from uniformly C^{14}-labeled fructose by the isolated rat diaphragm was approximately doubled by insulin and was almost completely abolished by glucose when present in a large concentration. The formation of CO_2 was also depressed by glucose, but not to a comparable degree.

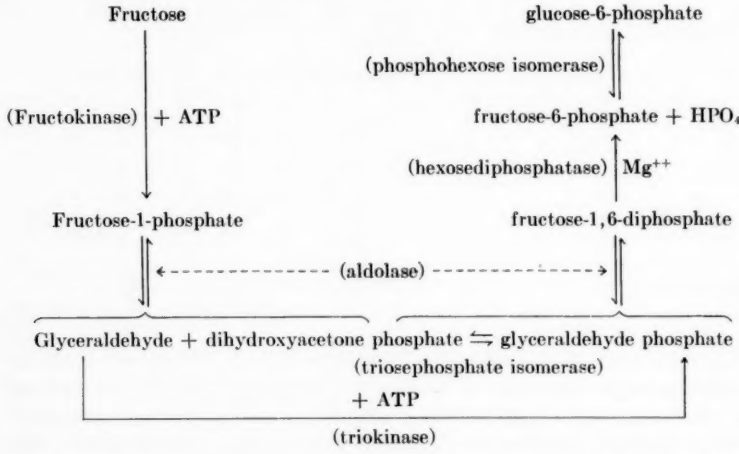
When fructose-1-C^{14} was used (Table IV), the newly formed glycogen contained more than 90 per cent of the total radioactivity in C-1, and a small but significant amount of the label in C-6. Apparently insulin did not affect the distribution pattern.

DISCUSSION

The finding that the glycogen formed in liver and muscle from glucose-1-C^{14} contains most of the isotope in position 1 confirms the data of Cook and Lorber (5) and provides a strong indication that glucose is built into the glycogen molecule essentially as an intact 6-carbon unit.

In contrast with glucose, fructose-1-C^{14} and sorbitol-1-C^{14} give rise, in the intact animal, to glycogen labeled in C-1 and C-6. The similarity between the results obtained with fructose and sorbitol is easily explained if it is admitted that the first step of the metabolism of sorbitol is an oxidation to fructose by the dehydrogenase described by Blakley (3). Fructose-1-C^{14} itself could be converted to fructose-6-C^{14} by way of mannitol. However, the metabolic inertness of this substance renders such a mechanism extremely unlikely and it may safely be concluded that a large fraction, at least of the fructose incorporated into liver and muscle glycogen in the intact animal, undergoes a preliminary splitting of the molecule into smaller fragments, very probably two 3-carbon units.

Applied to liver, this conclusion is in agreement with the data of Hers and Kusaka (1), who have shown fructose to be metabolized in liver extracts by the accompanying pathway.



This scheme provides a means whereby fructose-1-C^{14} can give rise to

glucose or glycogen labeled in C-1 and C-6, since it involves the formation of 3-C^{14}-dihydroxyacetone phosphate, which can be converted to 3-C^{14}-glyceraldehyde phosphate by the action of triosephosphate isomerase. It does not, however, allow any quantitative prediction to be made as to the relative radioactivity of the 2 carbons. In the event of a very slow triosephosphate isomerase reaction, most of the isotope would remain in carbon 1 of the resulting hexoses, whereas complete equilibration would be expected if the isomerization occurred sufficiently rapidly. The latter possibility is, however, rendered very unlikely by the results of Schambye *et al.* (18) who have found glycerol-1-C^{14}, which is believed to be metabolized via dihydroxyacetone phosphate, to give rise to glycogen labeled predominantly in carbon atoms 3 and 4, with 1.4 to 2.4 times more C^{14} in C-3 than in C-4. Such asymmetry should be even more favored in the present case, since unlabeled glyceraldehyde phosphate is fed into the system from the precursor molecule itself. There is therefore no need to assume the existence of a second direct pathway to account for the lack of isotope equilibration between C-1 and C-6.

The results obtained on muscle glycogen are much more difficult to interpret. The animal experiments give little information in this respect, since it is not known to what extent doubly labeled glucose, formed in the liver from fructose-1-C^{14}, has contributed to the formation of radioactive muscle glycogen. The data obtained with the isolated diaphragm (Table III) are consistent with the conclusion of Mackler and Guest (8) that the uptake of fructose by this preparation is mediated partly by an unspecific hexokinase and partly by fructokinase. The first mechanism, which is sensitive to insulin and inhibited by glucose, is believed to yield fructose-6-phosphate, whereas the other gives rise to fructose-1-phosphate. Under conditions known to favor the first process, fructose-1-C^{14} was found to give rise to glycogen labeled predominantly in position 1, with only 5 to 6 per cent of the radioactivity in carbon 6 (Table IV). It is therefore tempting to suppose that this mechanism leads to the formation of glycogen labeled exclusively in position 1 and that the second one alone is responsible for the appearance of C^{14} in position 6 of the glycogen. The involvement of fructose-1-phosphate does not, however, readily explain the labeling of carbon atom 6, as it does in liver, since muscle seems to lack triokinase and to contain a very active 1-phosphofructokinase (Table V). Thus conversion of fructose-1-phosphate to fructose diphosphate by muscle tissue does not seem to proceed by way of triose phosphates. However, the relative slowness of the hexosediphosphatase reaction may make it possible for the fructose esters to equilibrate with triose phosphates by means of reversible aldolase splittings.

Whatever the mechanisms involved in these processes, their physiologi-

cal significance remains doubtful. As pointed out by Mackler and Guest (8), the first is probably rendered largely inoperative by the presence of glucose, thus accounting for the lack of effect of insulin on the utilization of fructose *in vivo*. As to the other, although apparently supported by the isotope data obtained in the animal experiments, it is probably too slow to be of much importance. From what is known concerning the metabolism of fructose in the intact animal, it appears more likely that most of the labeled glycogen found in muscle after administration of radioactive fructose or sorbitol originates from glucose formed in the liver from the administered sugars.

It must finally be pointed out that there is no indication at all, in the experiments with glucose-1-C^{14}, of carbon atom 6 of the glycogen being

TABLE V

Enzymes Involved in Fructose Metabolism

Enzyme	Liver extract	Muscle extract
Fructokinase (19–22)*	Forms fructose-1-phosphate High affinity for fructose	Forms fructose-1-phosphate Very low affinity for fructose
1-Phosphofructokinase (19)	Not detected	Present
Triokinase (1)	Present	Not detected
Hexosediphosphatase†	Very active	Very slowly active

* Bibliographic reference Nos.

† Hers, H. G., unpublished data.

more radioactive than carbon atoms 2, 3, 4, or 5. This fact suggests that the tissues are incapable of converting glucose to fructose or fructose-1-phosphate to a significant extent. The possibility of reversible transformations between fructose-1-phosphate and glucose-6-phosphate, by isomerase or mutase types of reactions, can probably be excluded on these grounds.

SUMMARY

1. 1-C^{14}-labeled fructose, glucose, and sorbitol were injected into rats and the distribution of the labeling in liver and muscle glycogen was determined.

2. The liver glycogen formed from fructose-1-C^{14} and sorbitol-1-C^{14} contained approximately 55 to 60 per cent of the label in C-1 and 30 per cent in C-6. Similar values were found for muscle glycogen. In control experiments with glucose-1-C^{14}, most of the C^{14} was found in C-1 of the glycogen.

3. These data indicate that fructose is transformed to liver glycogen *in*

vivo by the pathway previously demonstrated by Hers and Kusaka (1) to occur *in vitro*, and that sorbitol is first oxidized to fructose rather than to glucose in the intact animal.

4. The formation of glycogen from fructose by the isolated rat diaphragm appears to occur mainly by the initial intervention of an unspecific hexokinase when no glucose is present and by that of a specific fructokinase when glucose is added. In the intact animal it seems probable that most of the glycogen arising from fructose in the muscle is formed after previous conversion of fructose to glucose in the liver.

I wish to thank Dr. W. Z. Hassid for constant interest and helpful advice during the course of this investigation.

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MANNITOL METABOLISM, A TRANSFERABLE PROPERTY OF PNEUMOCOCCUS

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The process of bacterial transformation offers a unique method of studying the relationship of the gene to its participation in the production of specific enzymes. Avery, MacLeod, and McCarty (1), in their studies on transformation in *Diplococcus pneumoniae*, showed that the transfer of heritable characters from a donor strain to a closely related strain was mediated by highly polymerized deoxyribonucleic acid (DNA) from the donor cells. The evidence that these and other transformations are essentially equivalent to the transfer of bacterial gene material from one cell to another has been presented elsewhere (2). It is reasonable to assume that the phenotypic expression of the character introduced into the transformed cells is due in part to an alteration of a specific enzymatic pattern of the cell. The DNA from the donor cell, once established in the genetic apparatus of the recipient cell, might be supposed to initiate the formation of enzymes related to the transformed property. The finding that the ability to utilize mannitol by a mutant strain of *D. pneumoniae* could be transferred to a mannitol non-utilizing strain offered a system amenable to enzymatic study.

Methods

Materials—The strains of pneumococcus used were rough variants of strain R36A (1) derived originally from type II. The wild type (M^-) does not utilize mannitol as a source of energy; the ultraviolet-induced mutant (M^+) does.

Cells to be used in respirometer studies or as sources of cell-free extracts were grown for 9 to 10 hours with a 7 per cent inoculum in a medium consisting of 1.0 per cent Bacto-casitone (Difco), 0.5 per cent Bacto-tryptone (Difco), 0.5 per cent NaCl, 0.1 per cent Bacto yeast extract (Difco), and 0.1 per cent sugar or sugar alcohol, and buffered with K_2HPO_4 (0.005 M). The cells were harvested by centrifugation at room temperature and washed once in 0.85 per cent NaCl.

The media and methods used in carrying out the genetic transfer of

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the mannitol-utilizing character are outlined in previous reports (3, 4). Briefly, the M^- recipient cells are grown in an albumin-containing growth medium treated with appropriate DNA from an M^+ donor strain, followed 5 or 10 minutes later by deoxyribonuclease to stop further action. The emergence of M^+ transformants is recognized by colony formation in a selective medium in which mannitol is the principal energy source. The medium is based upon a mixture of amino acids, vitamins, mannitol and sodium pyruvate, specific agglutinating anti-R globulin, mineral salts, and Bacto-tryptone (Difco), and supports continued growth of M^+ cells but not of M^- cells.

The partially defined medium used to study the adaptation of growing cells is similar to that already described (3). Diphosphopyridine nucleotide (DPN) (Worthington Biochemical) was 75 per cent pure. Reduced diphosphopyridine (DPNH) was prepared from it according to the method of Ohlmeyer (5). Triphosphopyridine nucleotide (TPN) was obtained from the Sigma Chemical Company (TPN "80").

Mannitol phosphate and mannose 6-phosphate were prepared according to the method described by Seegmiller and Horecker (6) for the chemical synthesis of glucose 6-phosphate. In the preparation of the mannitol phosphate, the phosphorylation of the mannitol with polyphosphoric acid was carried out for 10 hours. Under these conditions the final product was chiefly in the monophosphorylated state. Periodic acid oxidation and phosphorus analysis indicated that the product was 80 per cent in the form of a monophosphorylated primary ester.

Glucose 6-phosphate, glucose 1-phosphate, glycerophosphate, fructose 6-phosphate, fructose 1,6-diphosphate, and mannitol were commercial products. Fructose 1-phosphate synthesized by the method of Pogell (7) was provided by Mr. L. C. Mokrasch of the University of Wisconsin. Sedoheptulose 7-phosphate (75 per cent pure) was supplied by Dr. B. L. Horecker of the National Institutes of Health.

Catalase was obtained from the Armour Laboratories. Isomerase and yeast alcohol dehydrogenase were kindly supplied by Dr. M. Gibbs of Brookhaven National Laboratory. Glucose 6-phosphate dehydrogenase (*Zwischenferment*) was supplied by Dr. B. L. Horecker. Our thanks are due to each of the persons mentioned for their generous cooperation.

Cell-Free Extracts—Cell-free extracts were obtained by grinding washed, centrifuge-packed pneumococci with twice their wet weight of ground glass in a chilled mortar. After grinding the paste for 5 to 10 minutes, the mixture was taken up in water and centrifuged in the cold to remove the glass and cell débris. The straw-colored supernatant fluid was virtually free of whole cells.

Analytical Methods—Spectrophotometric measurements were made at

room temperature with a Beckman model DU spectrophotometer in 1.0 cm. cells. Manometric determinations were carried out with the conventional Warburg apparatus at 38°. Since hydrogen peroxide accumulates in the vessels under aerobic conditions, catalase was added to prevent its toxic effects. Protein was determined by the spectrophotometric method of Warburg and Christian (8), with a constant determined on bovine serum albumin, Fraction V (Armour).

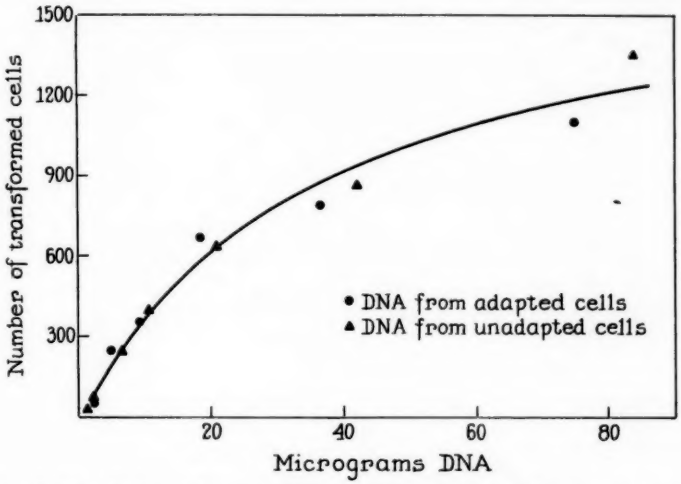

FIG. 1. Mannitol transformation by DNA extracted from cells grown in the presence and absence of mannitol.

Results

Genetic Transfer of Ability to Utilize Mannitol—When partially purified, high molecular weight DNA extracted from the mannitol mutant was added, under conditions appropriate for transformation, to the wild type, mannitol non-utilizing strain, some organisms were transformed with respect to mannitol utilization. The number of transformed cells could be scored quantitatively in a partially defined mannitol medium and shown to be dependent on the amount of highly polymerized DNA (Fig. 1). There is a direct proportionality at low concentrations and a leveling off at higher concentrations of DNA. This might indicate a limited number of effective DNA-receptor spots on the bacterial cell which become saturated at high concentrations of DNA. Since it had been shown that one of the enzymes initiating the metabolism of mannitol is present in mutant or transformed cells only when the cells are grown on the sugar alcohol (see

subsequent experiments), it was of interest to compare the transforming capacities of mutant cells grown in the presence and absence of mannitol. Fig. 1 shows that the DNA extracted from adapted cells has the same

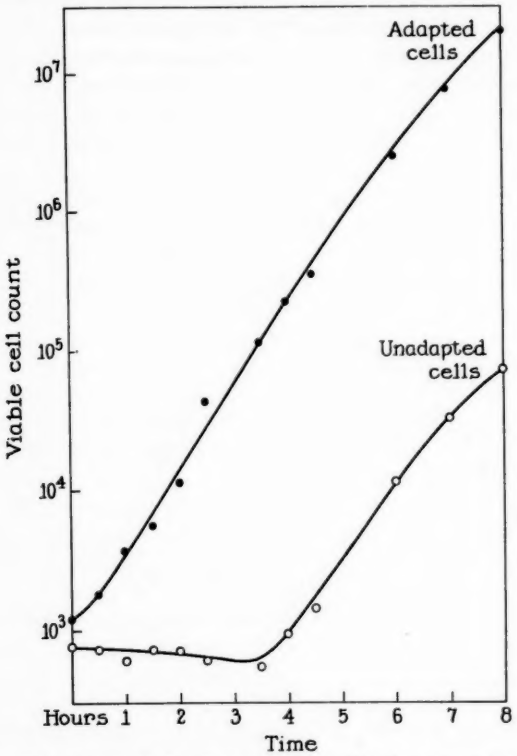

FIG. 2. Growth of mannitol-utilizing mutant, M+, in a mannitol medium (3). ○, inoculum grown in a medium containing glucose as energy source ("unadapted cells"); ●, inoculum grown in a medium containing mannitol as energy source ("pre-adapted cells").

transforming activity as comparable amounts of DNA extracted from cells grown in the absence of mannitol, as measured on an assay system with much lower sensitivity than that usually used (3, 4).

Inducible Nature of Mannitol-Utilizing Enzyme System—The growth curve of mannitol-utilizing mutants or transformants in a medium containing the sugar alcohol as a limiting energy source is dependent on the previous experience of the inoculating culture. Thus, when the mutant M+

was grown in a partially defined medium containing mannitol, its subsequent inoculation into a similar medium allowed the cells to continue to multiply at an exponential rate (Fig. 2). However, M^+ cells grown in a glucose medium, on inoculation into the mannitol medium, showed a pronounced lag of 3 to 4 hours before cell division was initiated. Once established, the growth rate of the glucose-grown inoculum ("unadapted") was the same as that of the mannitol-grown inoculum ("preadapted") in

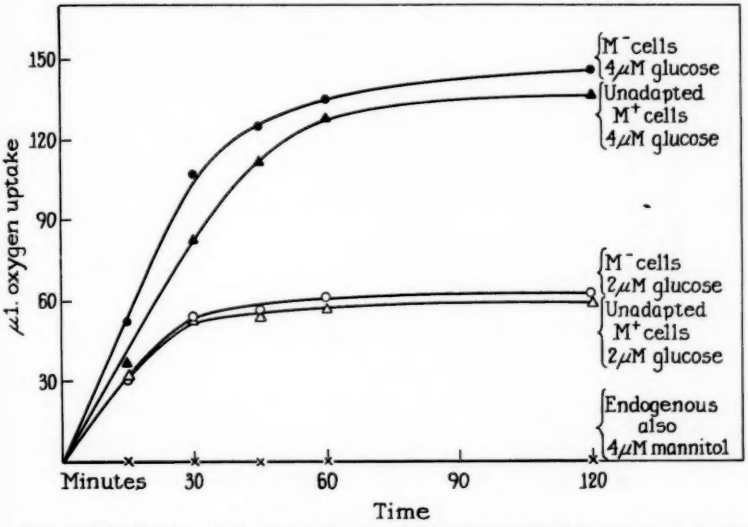

FIG. 3. Metabolism of glucose and mannitol by wild type, M^-, and mutant cells M^+, grown on a glucose medium. Experimental conditions: phosphate buffer, 0.02 M, pH 7.5; 20 mg. of wet weight cells per flask (about 2×10^9 viable cells); catalase 40 γ; total volume, 1.6 ml.; manometric technique, KOH in the center well; $t = 38°$. Circles, M^- cells; triangles, M^+ cells; crosses, M^- and M^+ cells.

the mannitol medium. It thus appears that the genetic capacity to use mannitol does not lead to optimal utilization of this sugar alcohol until the mechanism initiating mannitol metabolism has been induced by contact with the substance itself as the limiting energy source.

Like the ability to grow on mannitol, the ability to metabolize this compound in the respirometer is dependent both on the genetic endowment and the enzymatic state of the cell. Thus, resting cell suspensions of neither M^- nor M^+ previously grown in glucose-containing media will oxidize mannitol (Fig. 3). However, their oxidation of glucose is very similar.

Resting cell suspensions of the mannitol-utilizing mutant, M^+, do not

oxidize mannitol if the cells are grown in the absence of mannitol, although they will do so if grown on mannitol. However, when glucose-grown cells are added to mannitol in respirometer vessels in the presence of an external nitrogen and energy source, enzyme induction by the mannitol can be demonstrated (Fig. 4). Of all the constituents of refined and complex pneumococcal growth media so far tried, only a dilution of the relatively com-

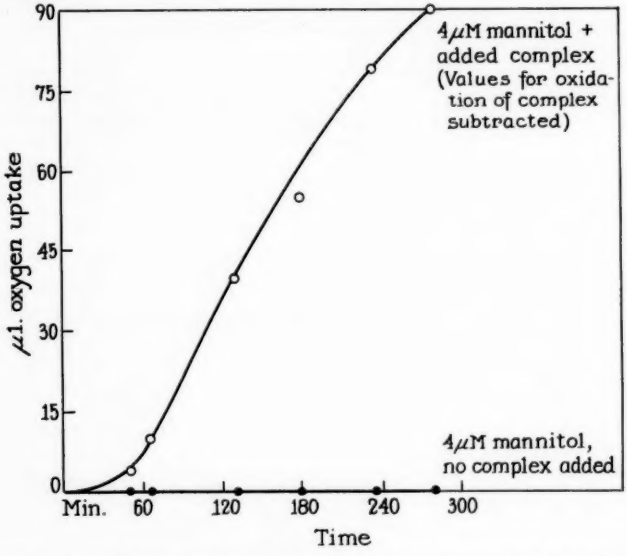

FIG. 4. Effect of the mannitol and nitrogen supply on mannitol oxidation by glucose-grown M^+. Experimental conditions: phosphate buffer, 0.02 M, pH 7.5; 20 mg. of wet weight glucose-grown cells per flask (approximately 3×10^9 viable cells); catalase 40 γ; added "complex," meat infusion plus neopeptone, 0.2 ml.; total volume, 1.6 ml.; manometric technique, KOH in center well; $t = 38°$. The cells were incubated with meat infusion plus neopeptone for 40 minutes in the respirometer flask before mannitol was added from the side arm.

plete mixture, meat infusion plus neopeptone ("complex" in Fig. 4), has regularly sufficed to allow adaptation in the respirometer. The wild type mannitol non-utilizing strain cannot oxidize mannitol even when the cells are grown in the presence of mannitol and a small amount of added glucose as energy source.

Products of Mannitol Metabolism by Whole Cells—When the mannitol mutant or transformant is grown in the presence of mannitol, the cells readily metabolize the sugar alcohol without any detectable lag period. The same cells do not oxidize the closely related sugar alcohol, dulcitol (Fig. 5). The

data show that, when oxygen consumption had ceased, 4 μatoms of oxygen had been consumed per micromole of mannitol and 3.12 μatoms of oxygen per μmole of glucose. If the oxidation of mannitol proceeded via a 6-carbon aldose or ketose, it would be expected that the oxygen consumed

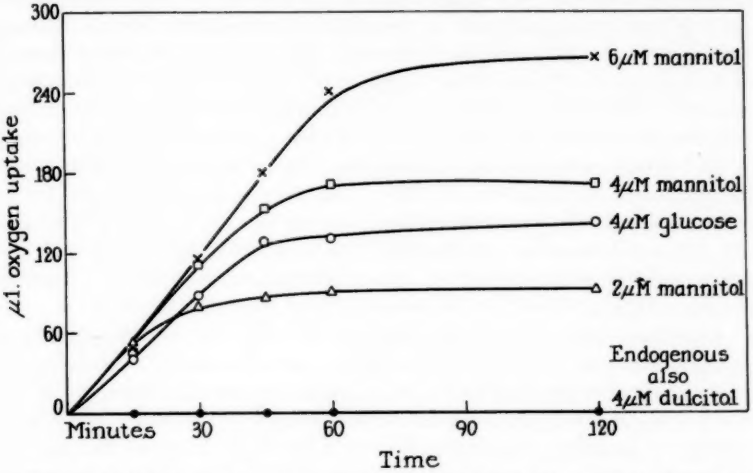

FIG. 5. Metabolism of glucose, mannitol, and dulcitol by mannitol-grown M^+. Experimental conditions: phosphate buffer, 0.02 M, pH 7.5; 20 mg. of wet weight adapted cells per flask (approximately 2×10^9 viable cells); catalase, 40 γ; total volume, 1.6 ml.; manometric technique, KOH in center well; $t = 38°$.

would be larger by an amount required to oxidize the sugar alcohol to a 6-carbon carbonyl compound.

$$C_6H_{14}O_6 + O \rightarrow C_6H_{12}O_6 + H_2O$$

Thus 1 μatom of oxygen would be required to convert mannitol to mannose or fructose.

The oxygen uptake data show that approximately 1 extra μatom of oxygen was required to oxidize mannitol presumably through its conversion to a 6-carbon carbonyl compound. The most likely oxidation product of mannitol is mannose if oxidation occurs at C-1, or fructose if oxidation occurs at C-2.

Studies with Cell-Free Extracts—In order to investigate more in detail the mechanism of mannitol metabolism and the enzymatic changes brought about as a result of mutation or transformation to mannitol utilization, studies were conducted with cell-free extracts of pneumococci.

Initial experiments showed that free mannitol was not metabolized by

cell-free extracts of mannitol-grown cells. Mannitol oxidation could not be detected in respirometer experiments; nor could the reduction of DPN or TPN be detected spectrophotometrically. The fact that whole cells grown in the presence of mannitol could metabolize mannitol, but that cell-free extracts did not, suggested that the mannitol is first phosphorylated before being oxidized. A mannitol phosphokinase would first be expected to convert mannitol to mannitol phosphate, which might then be dehydrogenated to form either mannose phosphate or fructose phosphate. However, attempts to obtain an active mannitol kinase preparation from cell-free extracts of mannitol-grown cells have thus far been unsuccessful.

Cell-free extracts from mannitol-grown cells will dehydrogenate mannitol phosphate in the presence of DPN. The course of this reaction can be followed spectrophotometrically by measuring the absorption at 340 mμ in the spectrophotometer. Difficulties resulting from the presence of systems oxidizing reduced DPN in the cell-free extracts could be eliminated by the addition of KCN or by purification of the crude extracts.

A Beckman quartz spectrophotometer was used to follow the activity of limiting amounts of mannitol phosphate dehydrogenase. The following compounds were pipetted into 1 cm. cuvettes, in a total volume of 3.0 ml.: 250 μmoles of tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.5, 20 μmoles of mannitol phosphate, 150 γ of DPN, and finally the enzyme preparation to be measured; the cuvette was kept at room temperature and readings were taken every 30 seconds for several minutes. When crude extracts were tested, 40 μmoles of KCN were added to prevent reoxidation of DPNH.

Partial purification of mannitol phosphate dehydrogenase from cell-free extracts of the mutant M^+ grown in the presence of mannitol was achieved as follows: M^+ cells grown in mannitol medium were ground with glass and extracted with 10 volumes of 0.85 per cent NaCl. After centrifugation for 20 minutes in the cold at 13,000 r.p.m. in an angle centrifuge, the supernatant fluid was collected and 0.2 ml. of 1 M $MnCl_2$ added to each 10 ml. of solution. After standing for 10 minutes, the precipitate was centrifuged at high speed. The supernatant fluid was dialyzed for several hours against distilled water. During the dialysis a precipitate formed which was centrifuged off at high speed. The clear supernatant fluid was then fractionated with saturated ammonium sulfate, brought to pH 7.5 with ammonium hydroxide. The precipitate formed between 50 to 80 per cent saturation was collected and dialyzed against distilled water for 2 hours. After dialysis, the preparation was treated with alumina gel Cγ. For each mg. of protein 1.3 mg. of the gel were added in several charges, the mixtures were centrifuged, and the precipitates discarded. The supernatant fluid of the last Cγ treatment yielded an active mannitol phosphate

dehydrogenase which had a specific activity 14 times that of the initial crude extract.

With limiting amounts of mannitol phosphate dehydrogenase, the reaction follows a zero order course and the rate of reduction of DPN is proportional to the amount of enzyme added (Fig. 6). 1 unit is defined as the amount of enzyme activity which causes an increase in optical density of 0.001 per minute at 340 mμ.

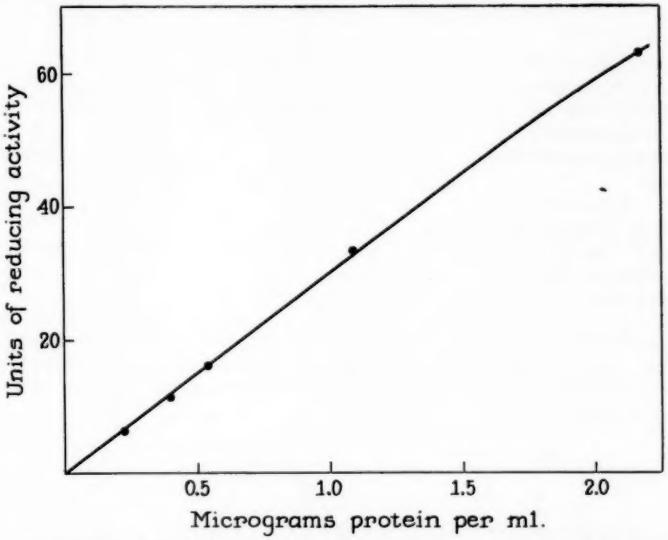

FIG. 6. Relation of protein concentration to enzyme activity. The conditions of the experiment are similar to those described in the text for following the purification of the mannitol phosphate dehydrogenase, except that 4 times the amount of DPN is added to each cuvette.

Oxidation of DPNH was also tested in the same system. The action of partially purified mannitol phosphate dehydrogenase in either direction is specific (Table I). No reduction of DPN or oxidation of DPNH was noted with fructose 1,6-diphosphate, mannose 6-phosphate, fructose 1-phosphate, or sedoheptulose 7-phosphate. The small amount of DPNH oxidation in the presence of glucose 6-phosphate and glucose 1-phosphate could be due either to contamination of these substrates with fructose 6-phosphate or to the conversion of the glucose phosphates to fructose 6-phosphate by enzymes present in the partially purified mannitol phosphate dehydrogenase. Small amounts of hexose isomerase have been detected in the partially purified extracts from pneumococcus.

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The pH optimum of mannitol phosphate dehydrogenase in Tris buffer is about 9.1 (Fig. 7). The activity of the partially purified extracts at various pH levels was determined by measuring the initial rate of reduction of DPN at 340 mμ.

TABLE I

Specificity of DPN-Linked Mannitol Phosphate Dehydrogenase

Substrate	Source of enzyme	ΔE_{340} DPN reduction	ΔE_{340} DPNH oxidation
Mannitol phosphate	Unadapted cells	0	
Fructose 6-phosphate	" "		0
Mannitol phosphate	Adapted cells	0.325	
α-Glycerophosphate	" "	0	
Fructose 6-phosphate	" "	0	0.270
Glucose "	" "	0	0.040
" 1-phosphate	" "	0	0.030
Fructose 1,6-diphosphate	" "	0	0
Mannose 6-phosphate	" "	0	0
Fructose 1-phosphate	" "		0
Sedoheptulose 7-phosphate	" "		0

Experimental conditions: Tris buffer, 0.083 M, pH 8.5; DPN, 0.6 mg.; DPNH, 0.17 mg.; mannitol phosphate dehydrogenase from adapted cells, 100 units; substrates, 0.0033 M; total volume, 3.0 ml.; t = room temperature; time of enzyme action, 5 minutes.

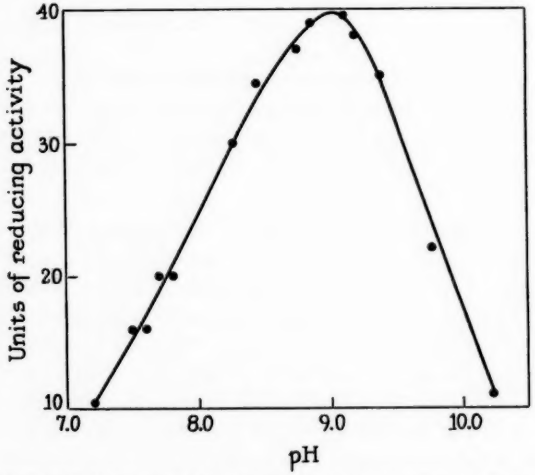

FIG. 7. Effect of pH on mannitol phosphate dehydrogenase. Experimental conditions are the same as those described in Fig. 6.

Oxidation Product of Mannitol Phosphate by Cell-Free Extracts—As has been already noted, the oxidation of mannitol phosphate by partially purified extracts of mannitol-grown pneumococci can be followed spectrophotometrically by measuring light absorption at 340 mμ. The equilibrium of the reaction can, however, be shifted toward the oxidized side by the addition of fructose 6-phosphate (Fig. 8). This apparent reversi-

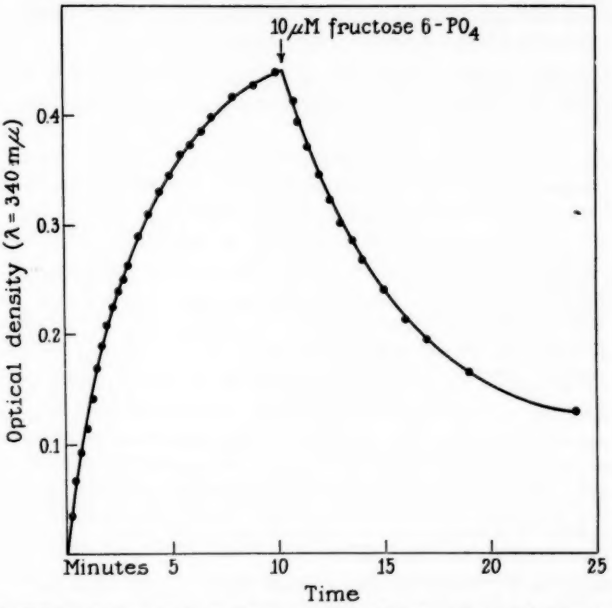

FIG. 8. Effect of fructose 6-phosphate on mannitol phosphate oxidation by mannitol phosphate dehydrogenase. Experimental conditions: Tris buffer, 0.083 M, pH 8.5; DPN, 0.6 mg.; mannitol phosphate, 4 μmoles; mannitol phosphate dehydrogenase, 1000 units; total volume, 3.0 ml.; t = room temperature.

bility suggests that fructose 6-phosphate is the oxidation product of mannitol phosphate. Mannose 6-phosphate does not reverse the reduction.

Fructose 6-phosphate was identified as the oxidation product of mannitol phosphate by coupling the system with purified enzymes of yeast and animal origin. The addition of hexose isomerase, *Zwischenferment*, and TPN to the reaction cell allowed conversion of the fructose 6-phosphate to glucose 6-phosphate and thence to 6-phosphogluconate, resulting in a reduction of TPN which can be followed spectrophotometrically at 340 mμ. The absorption due to DPNH formed in mannitol phosphate oxidation by

pneumococcal extracts could be eliminated by coupling with acetaldehyde and yeast alcohol dehydrogenase, which yielded alcohol and reoxidized the DPNH.

Mannitol phosphate + DPN^+ → fructose 6-phosphate + DPNH + H^+
Acetaldehyde + DPNH + H^+ → ethyl alcohol + DPN^+
Fructose 6-phosphate → glucose 6-phosphate
Glucose 6-phosphate + TPN^+ → 6-phosphogluconate + TPNH + H^+

Net reaction: mannitol phosphate + acetaldehyde + TPN^+ → 6-phosphogluconate + ethyl alcohol + TPNH + H^+

The complete system resulted in a reduction coupled with the enzymatic conversion of mannitol phosphate to 6-phosphogluconate (Table II).

TABLE II

Enzymatic Conversion of Mannitol Phosphate to 6-Phosphogluconate

	Observed ΔE_{340} in 13 min.
Complete system	0.222
Omitted DPN	0
" TPN and Mg^{++}	0.037
" *Zwischenferment* and isomerase	0.060
" mannitol phosphate	0
" " " dehydrogenase	0.003

Experimental conditions: The complete system contained the following substances in a final volume of 3.0 ml.: Tris buffer, 0.083 M, pH 8.5; acetaldehyde, 0.007 M; TPN, 200 γ; $MgCl_2$, 40 μmoles; DPN, 75 γ; *Zwischenferment*, 200 γ; partially purified mannitol phosphate dehydrogenase, 75 units; alcohol dehydrogenase, 80 γ; mannitol phosphate, 13 μmoles; t = room temperature.

The omission of TPN still allowed a small reading due to the fact that the reoxidation of DPNH by alcohol dehydrogenase and acetaldehyde is not complete. When isomerase and *Zwischenferment* are omitted, a small amount of reduction is noted. This is due to the presence of both these enzymes in the partially purified pneumococcus extracts. There is also a possibility that the pneumococcal extracts contain transdehydrogenase, the enzyme that catalyzes the direct transfer of hydrogen among the various oxidized and reduced pyridine nucleotides.

DISCUSSION

Mutations, genetic recombinations, and transformations among microorganisms are generally recognized by the changes brought about in the biochemical patterns of the altered cell. As a result of the findings with nutritional mutants of *Neurospora* by Beadle and Tatum (9), the concept

developed that the function of the gene, besides self-duplication, is that of influencing the synthesis of a particular enzyme (10). Transformations offer a direct approach to the biochemical study of the gene-enzyme relationship, since it appears that specific genes are being introduced into the recipient cells under controlled conditions (2).

The ability to utilize mannitol as a source of energy can be transferred to wild type, mannitol non-utilizing pneumococcal strains by highly polymerized DNA extracted from the utilizing mutants. The processes of both mutation and transformation result in the acquisition by the altered cells of the heritable ability to form the enzyme mannitol phosphate dehydrogenase. The enzyme is formed in the mutants or transformants only when the cells are grown in the presence of mannitol; it is DPN-linked, specific in its action, and dehydrogenates mannitol phosphate to form fructose 6-phosphate. The parent strain and non-adapted mutants or transformants do not show active dehydrogenase within the limits of sensitivity of measurement in the spectrophotometric method.

The assumption that mannitol is first phosphorylated before being oxidized is based on the findings that the sugar alcohol is metabolized by whole cells, but cell-free extracts can dehydrogenate only the phosphorylated form of mannitol. At present it is not known whether the transformed cell also acquires the ability to form mannitol phosphokinase, since it has not yet been possible to extract this enzyme from mannitol mutants, transformants, or wild type cells.

The mannitol transformation system provides a means of distinguishing between the rôle of the substrate and the gene in the formation of mannitol phosphate dehydrogenase. Whether or not the potentiality to make the dehydrogenase is expressed is determined by the presence or absence of mannitol as principal energy source. But either in its presence or absence this potentiality persists unchanged in the mutant strain, and is transferable from mother to daughter cell by the cellular genetic mechanisms, or to a non-mutant cell via the DNA in a transformation. The fact that DNA extracted from mutant cells either adapted or unadapted to mannitol has the same transforming efficiency per unit weight supports the classical genetic notions of stability of genes and their predominant rôle in enzyme determination. These findings supply further evidence of the relative independence of the genetic constitution from the enzymatic constitution, even in a substrate-dependent system.

The rôle of the substrate as inducer of enzyme formation comes into play only when the genetic potential, as in the mutant or transformant, is available in the cell. The formation of dehydrogenase has thus far been induced by mannitol only in the presence of a complex nitrogen source.

SUMMARY

Cells incapable of utilizing mannitol were in part transformed into cells with the ability to metabolize this sugar alcohol, by an extract of highly polymerized deoxyribonucleic acid (DNA) isolated from a mutant strain capable of metabolizing mannitol. As a result of this process, the transformed cells were able to form at least one new enzyme. This enzyme, mannitol phosphate dehydrogenase, is adaptive; *i.e.*, it is detected in the mutant or transformed cells only after exposure to mannitol in the growth medium. The enzyme is diphosphopyridine nucleotide-linked and specifically dehydrogenates mannitol phosphate to form fructose 6-phosphate. In spite of the great difference in enzyme content of substrate-adapted and -unadapted mutant cells, their DNA's are equally effective in bringing about the specific transformation, as would be expected of genetic material.

Addendum—Wolff and Kaplan have recently studied a DPN-linked enzyme system extracted from *Escherichia coli* cells which reduces fructose 6-phosphate to a hexitol monophosphate not fully identified at the time of the preliminary report (11). Through the kindness of these workers, it was ascertained that mannitol phosphate prepared by us behaves in a similar manner toward the *E. coli* system, as do reduction products prepared enzymatically or chemically and recognized as mannitol phosphates in their own work, a report of which will be forthcoming.[1]

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THE DESTRUCTION OF I^{131}-LABELED INSULIN BY RAT LIVER EXTRACTS*

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It is generally acknowledged that insulin is destroyed relatively rapidly in the intact organism (1–3). It has been demonstrated also that the biological activity of insulin is destroyed *in vitro* by homogenates, extracts, and slices of liver (4, 5). This destruction was attributed to an insulin-inactivating system which displays many of the characteristics of an enzyme system (4, 6). For the sake of convenience this system was designated as "insulinase."

In previous studies, the insulinase activity of homogenates or slices of liver was determined by assaying the hypoglycemic activity of the incubation mixture before and after a designated period of incubation (4). This procedure did not permit the accurate assay of small increments in the inactivation of insulin and consequently could not be utilized for studies on the mode of action or the specificity of insulinase. With the availability of I^{131}, however, it has become possible to label insulin and to trace the fate of the insulin molecule *in vivo* and *in vitro* (3, 7–9). Thus it was demonstrated that the insulin-inactivating activity of slices of rat liver parallels the degradation of the insulin molecule as reflected in an increase in the concentration of I^{131} in the non-protein fraction of the incubation mixture (9). The present study deals with the action of extracts of rat liver on labeled insulin and other similarly labeled proteins.

Method

Fed male rats, from 150 to 200 gm. in weight, were decapitated, and as much blood as possible was drained from the carcass of each animal. The liver was removed and immediately homogenized by the Potter-Elvehjem technique (10) with 4 volumes of 0.02 N sodium bicarbonate. After centrifugation for 30 minutes at 18,000 × *g* and 4°, the supernatant fluid was adjusted with M/15 phosphate buffer to the desired pH. Unless otherwise stated, the incubation mixture consisted of 1 ml. of extract plus 1 ml. of phosphate buffer containing a designated amount of a mixture of I^{131}-labeled and unlabeled protein. As a control, 2 ml. of buffer containing

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a similar quantity of I^{131}-labeled protein were incubated at the same time. At the end of the incubation period, 2 ml. of 10 per cent trichloroacetic acid were added to the incubation mixture. After centrifugation, but before separating the supernatant solution from the precipitate, the radioactivity of the total mixture was measured in a well type γ counter. Immediately thereafter, 3 ml. of the supernatant fluid were transferred to another test-tube, and the radioactivity was measured. Both counts were corrected for background and coincidence. The amount of protein degraded was calculated from the percentage of the total counts of the precipitated mixture that was found in the non-protein fraction and was expressed as units, mg., or mmoles of protein. Appropriate corrections were made for changes that occurred in some instances on incubation of the labeled protein without any extract.

The I^{131} insulin was prepared by subjecting crystalline insulin[1] which assayed 25 units per mg. to the procedure described by Talmage, Dixon, Bukantz, and Dammin (11). From 98 to 99 per cent of the I^{131} in the labeled insulin preparation was precipitable with 10 per cent trichloroacetic acid. The same procedure was utilized to label the following proteins: pepsin (twice crystallized),[2] chymotrypsin (crystalline, salt-free, from bovine pancreas),[3] lysozyme (crystallized three times, from egg white),[3] ribonuclease (crystallized five times),[3] human serum albumin,[4] and prolactin (6 units per mg.).[1] The molecular weights were assumed to be 12,000 for insulin, 15,000 for ribonuclease, 34,500 for pepsin, and 33,000 for prolactin.

Results

The extract employed in this study was the supernatant solution of a homogenate centrifuged at 18,000 $\times$ g for 30 minutes and presumably free of nuclei and cellular débris. Such an extract inhibits the hypoglycemic activity of insulin (6). This extract produces also a degradation of I^{131}-labeled insulin, as is reflected in the increase of the radioactivity of the non-protein fraction of the incubation mixture (Fig. 1). Likewise, a radioautograph[5] of the chromatogram of the non-protein fraction of the incubation mixture reveals that the insulin molecule is disrupted during the incubation with liver extract as contrasted with the lack of any effect on similarly labeled human serum albumin (Fig. 2).

It is evident also from Fig. 1 that with a concentration of 0.4 mg. of

[1] We are indebted to Eli Lilly and Company for generous supplies of crystalline insulin and for a preparation of prolactin.

[2] Purchased from the Worthington Biochemical Sales Corporation.

[3] Purchased from Pentex, Inc.

[4] Purchased from Armour and Company.

[5] We are indebted to Dr. Arthur Haelig for the radioautographs.

insulin per ml. of incubation mixture the degradation of insulin is linear during the first 30 minutes of incubation at 37° and pH 7.8.

The effect of pH on the degradation of insulin by the liver extract is illustrated in Fig. 3. If the increase in the radioactivity of the non-protein fraction is taken as an index of activity, it is apparent that maximal activity of the system responsible for the degradation of the I^{131} insulin occurs at pH 7.6 to 8.0.

That the degradation of I^{131} insulin is related to the quantity of enzyme present in the incubation mixture is revealed when various amounts of

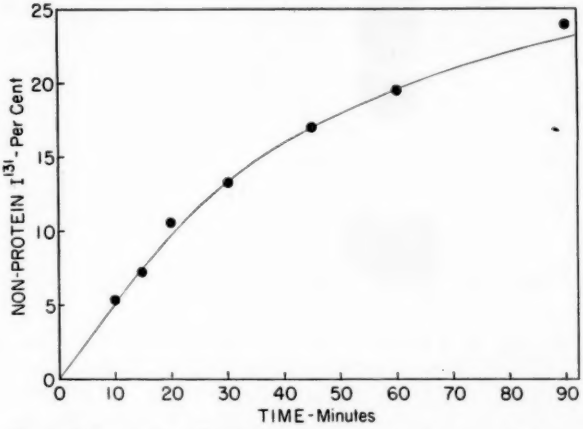

FIG. 1. Degradation of I^{131}-labeled insulin by liver extract. Per cent of total I^{131} found in non-protein supernatant fluid after incubation of 1 ml. of liver extract plus 1 ml. of phosphate buffer containing 0.8 mg. of I^{131} insulin at pH 7.8 and 37° for the designated period.

the centrifuged homogenate are incubated with the same quantity (0.8 mg.) of I^{131}-labeled insulin at pH 7.8 and 37° for 30 minutes (Fig. 4). Accordingly, the rate of release of I^{131} into the non-protein fraction of the incubation mixture under the specified conditions can serve as a measurement of the quantity of insulinase present. An insulinase unit may be defined as that quantity of enzyme which destroys 1 unit of insulin under the specified conditions. On that basis, the mean (± standard error) activity of extracts prepared daily from the livers of individual rats was found to be 4.69 ± 0.25 insulinase units per ml. of extract containing 18.5 ± 0.3 mg. of protein per ml.

The effect of substrate concentration on the rate of insulin destruction was determined at seven different concentrations ranging from 0.02 to 1.6

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mg. of I^{131} insulin per ml. of incubation mixture. Typical of eight such experiments are the data depicted in Fig. 5, where the initial velocities and the substrate concentrations are expressed as units of insulin (1 unit = 0.04 mg.) and plotted according to Lineweaver and Burk (12). Maximal

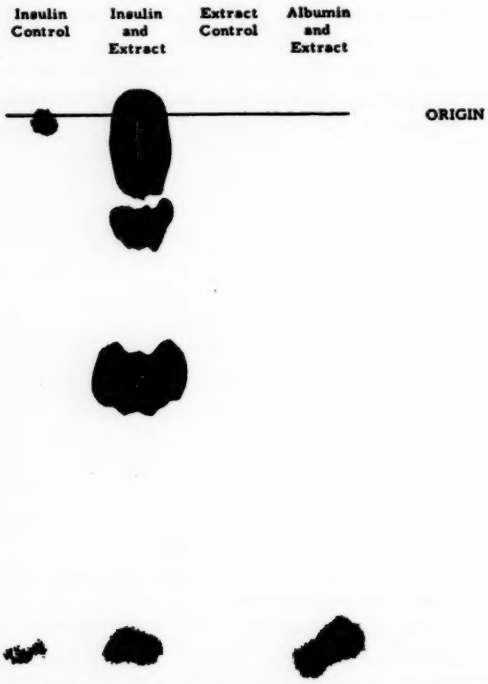

FIG. 2. Degradation of I^{131}-labeled insulin by liver extract. Radioautograph of descending chromatogram of trichloroacetic acid filtrates of I^{131} insulin, I^{131} insulin plus liver extract, liver extract alone, and I^{131} human serum albumin plus liver extract, after incubation at pH 7.8 and 37° for 30 minutes. The filtrates were placed along the top of the paper and developed for 24 hours in 2-butanol, 95 per cent ethanol, and 6 per cent ammonia (100:25:35).

velocity is achieved at a concentration of 10 units per ml. (0.4 mg.) of incubation mixture. Assuming a molecular weight of 12,000 and expressing the initial velocities as the millimoles of insulin destroyed per 30 minutes, the mean Michaelis constant, K_m, thus derived equals 8.5×10^{-3}, which is in the general range of values for many hydrolytic systems.

The effect of temperature on the rate of destruction of insulin is illustrated in Fig. 6. It is apparent that the activity rises with each incre-

ment in temperature until maximal activity is attained at 46°. Above 46°, the destruction of insulin falls off markedly, but about 30 per cent of the activity is still present at 80°. The mean temperature coefficient is 2.14 for the range 25–37° and 2.01 for 37–45°.

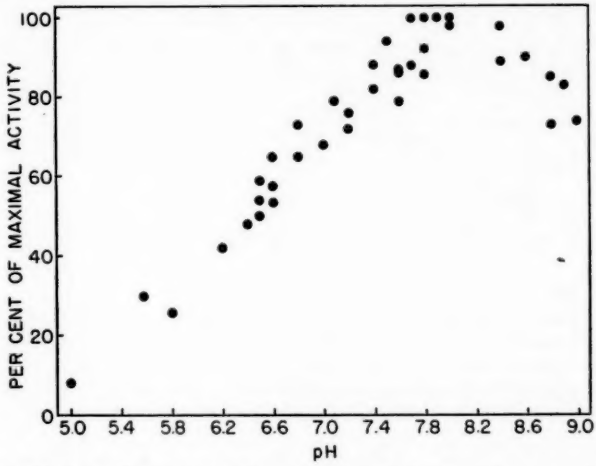

FIG. 3. Effect of pH on degradation of I^{131} insulin by liver extract. Incubation of 1 ml. of liver extract and 1 ml. of buffer containing 0.8 mg. of I^{131} insulin for 30 minutes at 37° and the designated pH. Phosphate buffer was used for pH 5 to 8.0 and Veronal buffer for pH 8 to 9.

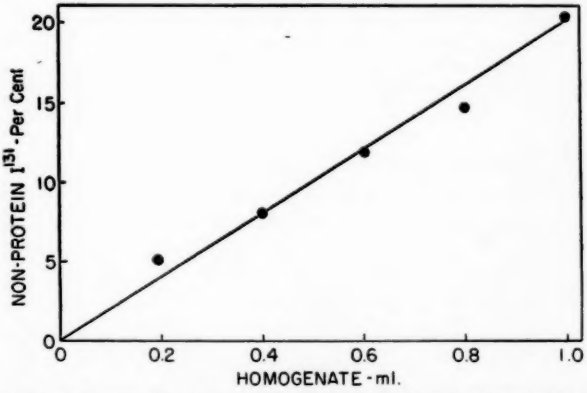

FIG. 4. Effect of quantity of liver homogenate on degradation of I^{131} insulin by liver extract. Various quantities of liver extract diluted to 1 ml. and incubated with 1 ml. of buffer containing 0.8 mg. of I^{131} insulin at pH 7.8 and 37° for 30 minutes.

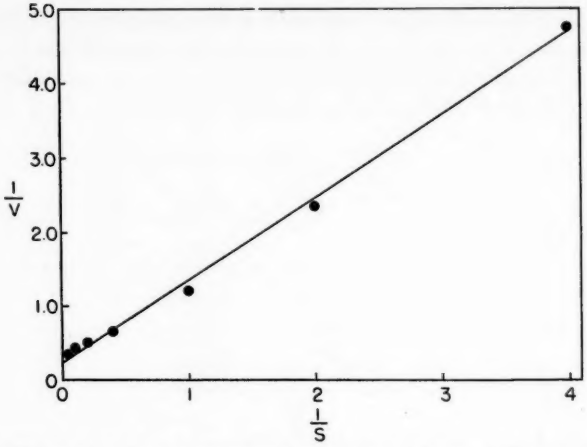

FIG. 5. Effect of substrate concentration on degradation of I^{131} insulin by liver extract. Incubation of 1 ml. of liver extract plus 1 ml. of phosphate buffer containing various quantities of I^{131} insulin, pH 7.8, for 30 minutes at 37°. V = units of I^{131} insulin degraded in 30 minutes, computed from increase in I^{131} in a trichloroacetic acid filtrate of incubation mixture. S = units of I^{131} insulin per ml. of incubation mixture.

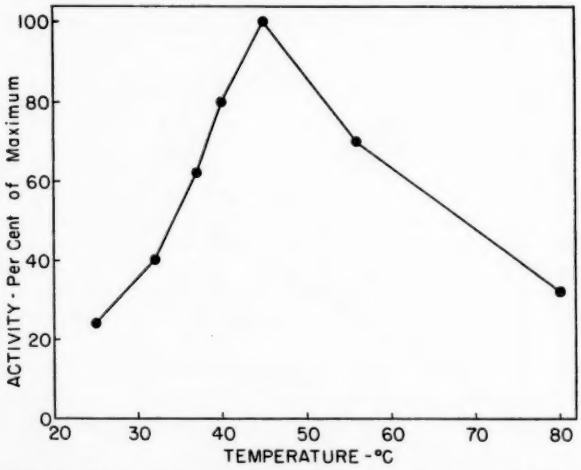

FIG. 6. Effect of temperature on degradation of I^{131} insulin by liver extract. Incubation of 1 ml. of liver extract plus 1 ml. of phosphate buffer containing 0.8 mg. of I^{131} insulin at pH 7.8 for 30 minutes at the designated temperatures.

In order to determine the rate of thermal inactivation of insulinase, experiments were performed in which extracts were heated at various temperatures for specified periods of time, cooled, and the residual activity assayed at 37° for 30 minutes. Fig. 7 illustrates such an experiment, in which the insulinase activity is expressed as the per cent of the activity of the unheated extract incubated at 37° for 30 minutes. When the extract is heated at 56° or higher for 5 or more minutes, approximately 70 per cent of the insulinase activity is lost. A longer heating period is required

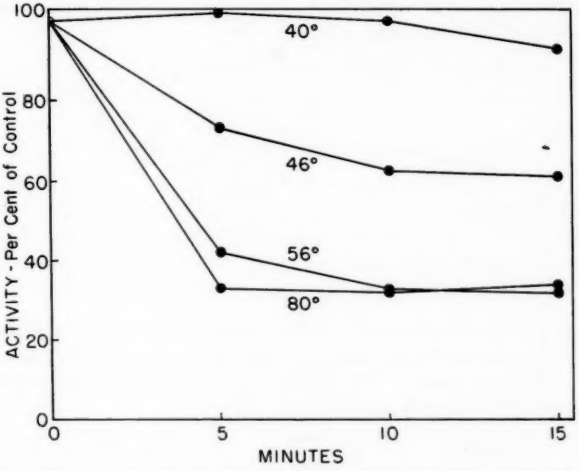

FIG. 7. Thermal inactivation of insulinase. 1 ml. of liver extract was heated at the designated temperature for the specified period of time, cooled, and incubated with 1 ml. of phosphate buffer containing 0.8 mg. of insulin at pH 7.8 at 37° for 30 minutes. Data expressed in terms of the activity observed with the unheated extract plus insulin incubated at 37°.

for inactivation of the enzyme at 46°. Essentially no inactivation occurs when the enzyme preparation is heated at 40° for 15 minutes.

The insulinase activity of the rat liver extract is almost completely removed from solution by precipitation of the proteins through adjustment of the extract to pH 4.2. Most of the activity is recovered from the precipitate obtained at pH 4.2 by redissolving the latter with 0.02 N sodium bicarbonate and adjusting to pH 7.8. For example, an extract with an initial activity of 3.73 units of insulinase per ml. (17.1 mg. of protein per ml.) was adjusted to pH 4.2, centrifuged, and the precipitate extracted with sodium bicarbonate and made up to the original volume. The activity of the solution of the precipitate at pH 4.2 was 2.9 units of insulinase

per ml. (3.4 mg. of protein per ml.), which represents a recovery of 77.8 per cent.

The degradation of insulin by rat liver extracts can be inhibited by a number of substances. The data in Table I indicate that the addition *in vitro* of small quantities of copper and zinc results in a marked inhibition

TABLE I

Effect of Inhibitors on Degradation of I^{131}-Labeled Insulin

0.8 mg. of labeled insulin incubated with 1 ml. of liver extract, pH 7.8, for 30 minutes at 37°. Rate of I^{131} released into non-protein fraction expressed as per cent of that released without inhibitor.

Inhibitor	Concentration	Rate
	M	
None		100
$CuSO_4$	5×10^{-4}	17.1
$ZnSO_4$	5×10^{-4}	4.5
p-Chloromercuribenzoate	5×10^{-4}	47.0
Iodoacetate	1×10^{-3}	43.0

TABLE II

Comparison of I^{131} Released from Labeled Insulin and Other Labeled Proteins

0.04 mM labeled protein incubated with 1 ml. of liver extract, pH 7.8, for 30 minutes at 37°. Rates of release of I^{131} into non-protein fraction expressed as per cent of that released from labeled insulin.

Substrate	Rate
Insulin	100
Ribonuclease	56.6
Pepsin	28.6
Prolactin	10.7
Lysozyme	0.7
Human serum albumin	0.4
Chymotrypsin	0

of insulinase activity. Likewise, the addition of *p*-chloromercuribenzoate and of iodoacetate inhibits the degradation of labeled insulin.

In order to determine the degree of specificity exhibited by the system responsible for the degradation of insulin, the action of liver extracts on various labeled proteins was studied (Table II). Although insulin is degraded to a greater degree than any other protein tested, an appreciable degradation of ribonuclease, pepsin, and prolactin occurs under similar conditions. The data illustrated in Fig. 8, however, indicate that the system responsible for the destruction of insulin is different from that re-

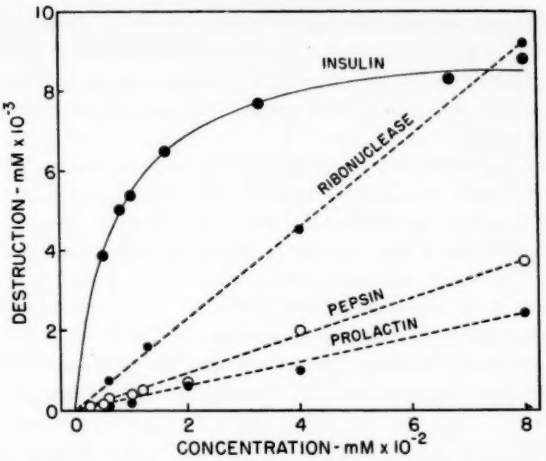

FIG. 8. Degradation of various I^{131}-labeled proteins by liver extract. Various concentrations of labeled insulin, ribonuclease, pepsin, and prolactin incubated with 1 ml. of liver extract at pH 7.8 at 37° for 30 minutes. Rate of destruction of protein calculated from increase of I^{131} in a trichloroacetic acid filtrate of incubation mixture.

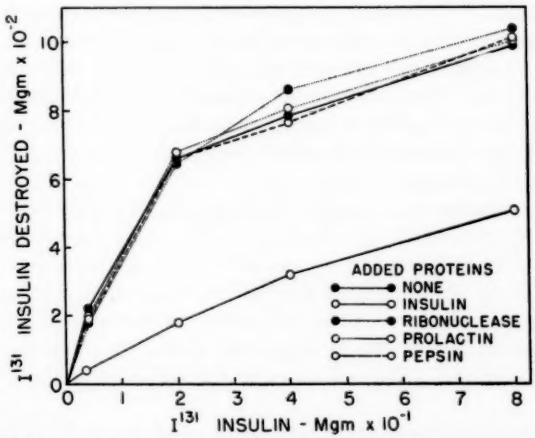

FIG. 9. Effect of added proteins on degradation of various concentrations of I^{131} insulin by liver extract. 1 mg. each of unlabeled insulin, ribonuclease, pepsin, and prolactin added to incubation mixture of 1 ml. of liver extract and 1 ml. of phosphate buffer containing various amounts of I^{131} insulin incubated at pH 7.8 and 37° for 30 minutes. Rate of destruction of the labeled insulin computed from the increase of I^{131} in the trichloroacetic acid filtrate of incubation mixture.

sponsible for the degradation of the other proteins. While the maximal velocity of insulinase is achieved at a concentration of from 0.02 to 0.03 mM insulin, the maximal activity of the systems responsible for the destruction of ribonuclease, pepsin, and prolactin is not achieved at a concentration of 0.08 mM.

Further evidence for the specificity of the system responsible for the destruction of insulin was gained from studies on the effect of various proteins on the release of I^{131} from I^{131}-labeled insulin. Fig. 9 depicts the data obtained when 1 mg. of each protein was added to different concentrations of I^{131} insulin and incubated with liver extract at pH 7.8 and 37° for 30 minutes. A Lineweaver-Burk calculation of these data reveals that, while a decrease of active enzymatic sites for the labeled insulin occurs with the addition of non-labeled insulin, the addition of ribonuclease, pepsin, or prolactin does not influence the velocity of the original labeled insulin degradation.

DISCUSSION

The data reported herein support the conclusions of previous studies that the liver of the rat contains an insulin-inactivating system (insulinase) (4, 5, 9). This system apparently inactivates insulin by catalyzing the hydrolysis of the insulin molecule by a reaction which essentially follows first order kinetics. The enzymatic nature of the insulin-inactivating system is suggested by the pH, temperature, and substrate concentration curves. In accord also is the fact that the insulinase activity is precipitated at pH 4.2. The thermal inactivation experiments suggest that either the insulinase is only partially inactivated by heat or that two distinct systems are present in the crude preparation, one that is heat-labile and the other that is heat-stable.

In previous studies, the activity of insulinase, as measured by the inactivation of the hypoglycemic effect of insulin, was shown to be inhibited by metals such as copper and by organic compounds such as iodoacetate. In accord are the studies reported herein. Consequently, it appears that a sulfhydryl group is essential to the activity of the system. Similar conclusions were reached by De Barbieri and Grassi (13) who demonstrated that oxidation of the —SH groups resulted in an inactivation of insulinase activity.

Previous studies revealed that extracts capable of inactivating the biological activity of insulin were without any proteolytic effect on denatured hemoglobin at pH 7.5, while some tissues rich in proteinases were poor in insulinase activity (6). Consequently, it was concluded that the insulinase activity of liver extracts or slices is not due to a non-specific proteolytic enzyme. The present study reveals that, whereas human serum albumin,

lysozyme, and chymotrypsin are not degraded by liver extracts at pH 7.8, other proteins of relatively small molecular weight may undergo appreciable degradation under similar conditions. The data indicate, however, that the system responsible for the destruction of insulin is different from that responsible for the destruction of ribonuclease, pepsin, or prolactin. Accordingly, it would appear that insulinase is a relatively specific enzyme which is responsible for the destruction of insulin. Similar conclusions were reached by Tomizawa *et al.* (14) in a preliminary note which appeared while this report was in preparation.

SUMMARY

The effect of a crude extract of rat liver on the degradation of I^{131}-labeled insulin was measured by determining the proportion of the protein-bound I^{131} which appeared in the non-protein fraction of the incubation mixture under specified conditions. Several properties of the system responsible for the degradation of I^{131} insulin, such as pH-activity curves, temperature curves, effect of substrate concentration, and rate of thermal inactivation, have been described.

The specificity of the system responsible for the destruction of insulin (insulinase) was determined by comparing the effect of the liver extract on I^{131} insulin with that on similarly labeled ribonuclease, pepsin, prolactin, lysozyme, chymotrypsin, and human serum albumin. Under conditions which yielded maximal degradation of insulin no appreciable effect on lysozyme, chymotrypsin, or human serum albumin was noted. Under similar conditions, however, an appreciable degradation of ribonuclease, pepsin, and, to a lesser degree, prolactin occurred. Studies on the effect of substrate concentration on the degradation of the different proteins and of the addition of these proteins on the degradation of several concentrations of insulin indicate that the destruction of insulin is due to a specific system (insulinase).

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CRYSTALLINE TRANSKETOLASE FROM BAKERS' YEAST: ISOLATION AND PROPERTIES*

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The wide-spread occurrence and the rôle of an enzyme which catalyzes the production of triose phosphate from pentose phosphate have been reviewed elsewhere (1–3). This enzyme has been crystallized from yeast and has been named transketolase (4). Investigations with this enzyme have shown that (*a*) ribulose-5-phosphate is a substrate cleaved by this enzyme; (*b*) the reaction occurs only in the presence of aldehydes which act as acceptors for the ketol group of ribulose-5-phosphate; (*c*) the products of the cleavage of ribulose-5-phosphate are glyceraldehyde-3-phosphate and a keto sugar whose nature depends upon the acceptor aldehyde present (*e.g.*, in the presence of ribose-5-phosphate a heptulose phosphate is formed); (*d*) the enzyme also catalyzes the decarboxylation of hydroxypyruvate in the presence of an acceptor aldehyde (*e.g.*,with glyceraldehyde-3-phosphate as acceptor a ketopentose is formed which appears to be ribulose-5-phosphate); (*e*) the enzyme requires thiamine pyrophosphate and magnesium ions for activity.

It is the purpose of this paper to describe the purification and crystallization of transketolase from bakers' yeast, and to report in detail experiments carried out with the purified enzyme.

Materials, Methods, and Preparation of Enzymes

Materials—Fructose-6-phosphate, fructose-1,6-diphosphate, adenosine-5-phosphate, and yeast nucleic acid were obtained from the Schwarz Laboratories, DL-glyceraldehyde, DL-glyceraldehyde-3-phosphate, and bromopyruvic acid from the Concord Laboratories, and protamine sulfate from the Nutritional Biochemicals Laboratory. Ribose-5-phosphate was prepared from adenosine-5-phosphate as described previously (5), and also from ribose, according to Michelson and Todd (6). Ribulose-5-phosphate,

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sedoheptulose-7-phosphate, L-erythrulose *o*-nitrophenylhydrazone, and sedoheptulosan were kindly supplied by Dr. B. L. Horecker. Potassium hydroxypyruvate was prepared from bromopyruvic acid (7). DPN[1] was obtained from the Pabst Laboratories, and DPNH was prepared by the method of Ohlmeyer (8). TPN of about 80 per cent purity was purchased from the Sigma Chemical Company. Glycolaldehyde was prepared from dihydroxymaleic acid by the method of Fischer and Taube (9). ThPP was obtained from the Merck Laboratories. Glucose-6-phosphate was prepared according to the method of Levene and Raymond (10).

Methods—For the determination of keto sugars, the method of Dische and Borenfreund (11) was used. Pentose was determined by the orcinol reaction (12), and heptuloses were determined as described by Dische (13). Hydroxypyruvate was assayed with DPNH and lactic dehydrogenase. Glucose-6-phosphate was determined with TPN and glucose-6-phosphate dehydrogenase purified from brewers' yeast (14).

Preparation of Enzymes—Seven times recrystallized triosephosphate dehydrogenase free of triose isomerase was kindly supplied by Dr. M. V. Simpson. L-α-Glycerophosphate dehydrogenase, aldolase, triosephosphate isomerase, and lactic dehydrogenase were prepared as described previously (5, 15).

Assay Method for Transketolase

The assay is based upon the oxidation of DPNH, followed at 340 mμ with a model DU Beckman quartz spectrophotometer. In the presence of a mixture of ribose-5-phosphate, ribulose-5-phosphate, triosephosphate isomerase, and glycerophosphate dehydrogenase, the oxidation of DPNH follows the kinetics of a zero order reaction on addition of limiting amounts of transketolase. A unit of enzyme is defined as that amount of enzyme which gives an optical density change of 0.001 per minute under the conditions of the assay. Specific activity is expressed as units of enzyme activity per mg. of protein. Protein concentration was determined spectrophotometrically (16); in the presence of nucleic acid the biuret test was used (17). As seen in Fig. 1, proportionality between enzyme concentration and activity was obtained.

Procedure—Into a quartz micro cell with a 1 cm. light path the following reagents were pipetted: 0.6 ml. of distilled H_2O, 0.05 ml. of 0.5 M glycylglycine buffer, pH 7.6, 0.05 ml. of 0.002 M DPNH, 0.05 ml. of crude glycerophosphate dehydrogenase (10 mg. per ml.), 0.05 ml. of the ammonium

[1] The following abbreviations will be used in the text: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ThPP, thiamine pyrophosphate; HDP, fructose-1,6-diphosphate; TK, transketolase; EDTA, ethylenediaminetetraacetate (Versene).

salt of isomerase product solution (see below), 0.05 ml. of 0.1 per cent ThPP, 0.05 ml. of 0.06 M $MgCl_2$, and finally 0.05 ml. of a transketolase solution containing 500 to 1000 units per ml. Due to the presence of traces of transketolase in the muscle preparation, readings are taken every 30 seconds for several minutes before the addition of transketolase, and the blank observed is subtracted from the activity obtained upon the addition of transketolase.

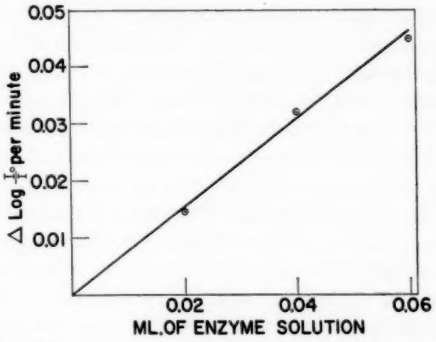


FIG. 1. Proportionality between activity and concentration of transketolase. The details of the assay are described under "Assay method for transketolase."

Preparation of Pentosephosphate Isomerase and Isomerase Product

Pentosephosphate Isomerase

A crude bakers' yeast extract was fractionated with acetone as described below for the preparation of transketolase to obtain Acetone Fraction II (33 to 50 per cent acetone). This fraction was suspended in 100 ml. of distilled water and dialyzed at 0° against 25 to 50 volumes of distilled water for 12 hours, the water being changed once during the process. The preparation was then centrifuged at 18,000 × *g* at 5°. The supernatant solution was diluted 1:5 with distilled water at 60°, and the temperature of the mixture was rapidly brought to 60° and maintained there for 25 minutes. After cooling to 5–10° and centrifuging at 5°, 29 gm. of ammonium sulfate were added per 100 ml. of supernatant fluid at 5°. The suspension was centrifuged and the precipitate discarded. To every 100 ml. of supernatant fluid 16.7 gm. of ammonium sulfate were added, the suspension was centrifuged, and the precipitate dissolved in 50 ml. of distilled water. This preparation of isomerase was now virtually free of transketolase and was stored at −20°. Before use it was dialyzed against cold distilled water until free of sulfate.

Isomerase Product—The equilibrium mixture of ribose-5-phosphate and

ribulose-5-phosphate (isomerase product) was prepared as follows: 1 gm. of the barium salt of ribose-5-phosphate was dissolved in 10 ml. of 0.1 N HCl. This solution was heated in a boiling water bath for 4 minutes, cooled, and the Ba^{++} removed with a slight excess of ammonium sulfate. The solution was neutralized to pH 7.6, 4 ml. of dialyzed isomerase were added, and the volume made up to 20 ml. with water. The mixture was incubated at 38° and the formation of keto sugar was followed by the carbazole reaction until no further increase in ketopentose was noted. About 2 to 3 hours were usually required for completion of the reaction. 3 ml. of 50 per cent trichloroacetic acid were then added, and, after centrifugation, the supernatant fluid was adjusted to pH 8.0, 5 ml. of 1 M barium acetate were added, and the mixture was centrifuged. The precipitate was washed with water, the washings and supernatant fluid were combined, and 4 volumes of 95 per cent ethanol were added. After 2 to 3 hours at 0°, the precipitate was collected by centrifugation, washed once with absolute ethanol, and dried in a vacuum desiccator. The barium salt was stored in a desiccator at 2°. A solution of the ammonium salt was prepared by treating a 3 per cent solution of the barium salt with a slight excess of ammonium sulfate and removal of the barium sulfate by centrifugation. The solution, adjusted to pH 6.5, could be stored at −20° for several days.

Oxidation of Isomerase Product with Iodine—To 3 ml. of the solution of the ammonium salt of isomerase product, 5.8 ml. of 0.1 N iodine were added. Then 0.1 N NaOH was added until a pH of 8.5 was reached, and the pH was then maintained at 8.5 by addition of alkali over a period of 40 minutes, at which time the rate of acid production was very slow. 1.5 ml. of 1 M barium acetate and 4 volumes of 95 per cent ethanol were then added. The suspension was stored at 0° for 1 hour and then centrifuged. The precipitate was washed twice with 95 per cent ethanol and once with ether, and stored in a desiccator at 2°. According to a carbozole assay of this material, no loss of the ketopentose occurred during the oxidation.

Preparation of Crystalline Transketolase from Bakers' Yeast

Dried bakers' yeast was used as starting material. This was prepared by crumbling and spreading fresh 1 pound cakes between sheets of paper and drying at room temperature for a period of 4 days. The dried yeast was kept at 5° in stoppered bottles.

300 gm. of dried bakers' yeast were extracted with 900 ml. of 0.066 M disodium phosphate with occasional stirring at 37° for 2 hours. The extraction was continued at room temperature for an additional 3 hours, and an extract was obtained by centrifuging at 3000 r.p.m. for 80 minutes. (Assays of transketolase in the crude extract by the method outlined above

are inaccurate and represent approximate values.) The residue was re-extracted (after storage at 0° overnight) with 600 ml. of 0.066 M disodium phosphate at 37° for 2 hours and at room temperature for 1 hour. The extract obtained after centrifugation was adjusted to pH 6.5 before proceeding to acetone fractionation. Yield and specific activity of transketolase in the second extract were as good as or better than in the first extract.

Acetone Fractionation—0.5 volume of ice-cold acetone was added to the crude extract at −2° in a dry ice-alcohol bath. The mixture was centrifuged for 15 to 20 minutes at −2° and the precipitate discarded. To the clear supernatant solution another 0.5 volume (per volume of original extract) of acetone was added, and the precipitate collected by centrifugation and dissolved in 100 to 150 ml. of cold distilled water. After dialysis for 12 hours at 5° against 25 to 50 volumes of distilled water, the precipitate was removed by centrifugation.

Alcohol Fractionation—The pH of the supernatant solution was adjusted to 6.5 to 6.6 with 1 N acetic acid. An equal volume of 95 per cent ethanol was added over a period of about 30 minutes, the temperature of the mixture being kept at −6° in a dry ice-alcohol bath. After centrifuging at −6°, the supernatant solution was discarded. The precipitate was suspended in 60 ml. of water and left at room temperature for 10 minutes. After centrifuging in the cold, the precipitate was discarded. The supernatant solution was refractionated with alcohol as above (at pH 6.5 to 6.6) to a final concentration of 25 per cent. After centrifuging at −6°, the precipitate was dissolved in 20 ml. of water (Alcohol Fraction II).

Nucleic Acid Fractionation—The pH of Alcohol Fraction II was adjusted to 5.5 with 0.1 N acetic acid at 5–10°, centrifuged at 0°, and the precipitate discarded. To the supernatant solution (at pH 5.5) 0.39 ml. of 5 per cent sodium nucleate was added for every 100 mg. of protein. After adjusting the pH to 5.0 with 0.1 N acetic acid, the mixture was centrifuged at 0° and the supernatant fluid discarded. The protein nucleate precipitate was dissolved in 10 ml. of water by cautiously adjusting the pH to 7.2 with 0.1 N NaOH at 5–10°. The protein solution was diluted to contain 20 to 25 mg. per ml. The pH was adjusted to 5.3 to 5.4 with 0.1 N acetic acid, the mixture centrifuged at 0°, and the precipitate dissolved in 5 ml. of water by adjusting the pH to 7.2. The supernatant solution was adjusted to pH 5.0, centrifuged at 0°, and the precipitate dissolved in 5 ml. of water by adjusting the pH to 7.2. These two fractions were assayed for transketolase activity, most of which was usually in the precipitate at pH 5.3. Transketolase is stable for weeks at this point if kept at 5°.

Removal of Nucleic Acid with Protamine Sulfate—The protein nucleate solution was adjusted to pH 6.7. A 6 per cent solution of protamine sul-

fate was adjusted to pH 6.5 and heated to 50°. For every 100 mg. of protein, 0.28 ml. of this solution was added. The precipitate was centrifuged and discarded. The pH of the supernatant solution was adjusted to 7.2 with 0.1 N NaOH and recentrifuged at 0° if turbid.

Alumina Gel Adsorption of Impurities—To the supernatant solution at room temperature, 0.1 volume of alumina Cγ (18), containing 16 mg. of dry weight per ml., was added. After stirring occasionally for 5 minutes at room temperature, the suspension was centrifuged and the supernatant solution assayed. The alumina Cγ treatment was repeated several times until no more than 20 per cent of transketolase was lost. If little increase in purity was observed, the alumina Cγ treatment was repeated after adjusting the pH of the protein solution to 5.7 to 5.8 with 0.1 N acetic acid.

Ammonium Sulfate Fractionation and Crystallization of Transketolase—To the solution, saturated ammonium sulfate was added slowly at 5–10° to a final saturation of 50 per cent. After standing for a few hours at 0°, the precipitate was removed by centrifugation at 16,000 × *g* at 0°. The precipitate, which contained less than 10 per cent of the total activity, was discarded. Saturated ammonium sulfate was then added to the 50 per cent saturated supernatant solution until 70 per cent saturation was reached. After standing several hours at 0°, the solution was centrifuged in the cold at 16,000 × *g* and the precipitate dissolved in cold water to give a final protein concentration of 5 to 10 mg. per ml. The solution of this precipitate was assayed to determine the recovery of transketolase. If recovery was not complete, the ammonium sulfate concentration of the supernatant solution was raised to 80 per cent saturation. To the solution of the precipitated enzyme, saturated ammonium sulfate was then added slowly with stirring until slight turbidity was visible (50 to 60 per cent saturation). Crystallization of the enzyme was allowed to take place at 5° overnight. Next day the crystals were centrifuged and recrystallized as above. It was necessary to recrystallize several times to remove contaminating pentosephosphate isomerase. A representative protocol of the purification procedure is presented in Table I. Frequently the specific activity of the acetone and alcohol fractions was considerably higher than indicated in Table I.

A modification of the above procedure of ammonium sulfate fractionation and crystallization has been used more recently, resulting in better yields and higher specific activity of the enzyme. Saturated ammonium sulfate at pH 7.6 (the pH was adjusted with concentrated ammonium hydroxide and determined with a glass electrode on a 1:50 dilution of the saturated solution) was used and advantage taken of the negative temperature coefficient of solubility exhibited by this enzyme in ammonium sulfate by allowing the enzyme to crystallize slowly at room temperature.

TABLE I

Purification of Transketolase from Bakers' Yeast

Fraction	Total units of enzyme	Specific activity
		units per mg. protein
Crude extract		
Acetone Fraction II	1,032,000	133
Alcohol " "	800,000	643
Supernatant, pH 5.5	893,000	1,240
Protein nucleate, pH 5.5–5.4	738,400	3,466
After protamine treatment	576,000	8,000
" 2 treatments with alumina C_γ	576,000	25,925
$(NH_4)_2SO_4$, crystals at 59% saturation	280,000	50,000
Recrystallization I	273,000	54,400
" II	184,000	54,000
Alkaline ammonium sulfate procedure		
Protein nucleate, pH 5.5–5.0	12,560,000	4,608
After protamine treatment	12,970,000	6,143
" 2 treatments with alumina C_γ	10,080,000	7,347
$(NH_4)_2SO_4$, 59% ppt.	4,419,000	30,000
" refractionated 45–50% saturation	4,400,000	119,000
" crystals at 50% saturation	2,160,000	148,000
" supernatant	2,080,000	134,400

FIG. 2. Photomicrograph of crystalline transketolase (specific activity = 150,000). The crystals were stained with methylene blue; magnification, 1600 ×.

XUM

The enzyme obtained by this procedure requires ThPP, and specific activities as high as 150,000 have been obtained[2] (see Table I). Crystals thus obtained and stained with methylene blue are shown in Fig. 2.

Properties of Crystalline Transketolase

ThPP Requirement—Partially purified preparations of yeast transketolase show little or no activation by ThPP. However, prolonged dialysis against a solution of 0.6 per cent EDTA-0.9 per cent KCl, pH 7.4 (4), resulted in a loss of transketolase activity which was fully restored by addition of both magnesium and ThPP. ThPP can be more readily re-

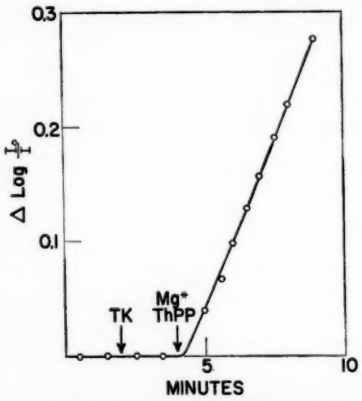


FIG. 3. Thiamine pyrophosphate requirement of crystalline transketolase. Components in this system are described under "Assay method for transketolase," 0.4 γ of transketolase (specific activity = 150,000) was added where indicated.

moved by fractionation with alkaline ammonium sulfate (see "Purification of transketolase," Table I). The activity of transketolase, crystallized from alkaline ammonium sulfate, in the presence and absence of ThPP, is shown in Fig. 3. A requirement for Mg^{++}, as observed after dialysis against EDTA-KCl, was not obtained by this procedure, but a definite lag of triose phosphate formation was observed in the absence of Mg^{++}.

Stability—Suspensions of crystalline transketolase are stable in alkaline ammonium sulfate, even at room temperature. Water solutions of transketolase lose activity but are stable over a period of several weeks in the ice box in 0.008 M glycylglycine buffer at pH 7.4.

[2] A preparation of transketolase with a specific activity of 150,000 units per mg. of protein was analyzed by Dr. I. C. Gunsalus and found to contain a negligible amount of lipoic acid.

Substrates and Products of Transketolase-Catalyzed Reactions

Pentose Phosphates—Crystalline transketolase, free of pentosephosphate isomerase, did not form triose phosphate with either ribose-5-phosphate or ribulose-5-phosphate as substrate. However, with a mixture of both (isomerase product), formation of triose phosphate could be readily demonstrated. Although fresh solutions of ribose-5-phosphate are essentially free of the keto sugar, it is difficult to obtain preparations of ribulose-5-phosphate which are free of the aldo sugar. As can be seen in Fig. 4, on addition to the assay system of ribulose-5-phosphate or isomerase product which has been subjected to iodine oxidation, the reaction proceeded until the residual ribose-5-phosphate was depleted. Upon further addition of an acceptor aldehyde (*e.g.* ribose-5-phosphate or glyceraldehyde), the for-

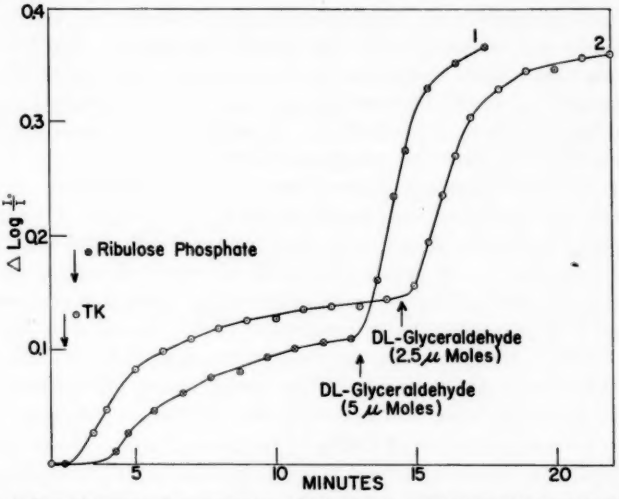


FIG. 4. Requirement for acceptor aldehyde in splitting ribulose-5-phosphate. Curve 1, ribulose-5-phosphate (Dr. B. L. Horecker); Curve 2, isomerase product subjected to iodine oxidation. All components except substrate, acceptor aldehyde, and transketolase were added in amounts given under "Assay method for transketolase." Curve 1, transketolase (2600 units, specific activity = 16,000) was added at zero time and ribulose-5-phosphate as shown in the graph. 5 μmoles of DL-glyceraldehyde were added after the reaction had come essentially to completion. Curve 2, iodine-treated isomerase product (0.12 μmole of pentose) was added at zero time. Transketolase (1200 units, specific activity = 14,500, not requiring ThPP) was added where indicated. 2.5 μmoles of DL-glyceraldehyde were added where indicated. The preparations of TK used in the above experiments contained traces of pentose isomerase activity, responsible for the slow reaction before addition of acceptor aldehyde.

mation of triose phosphate was resumed. The presence of ribose-5-phosphate in preparations of ribulose-5-phosphate may well explain experiments in which ribulose-5-phosphate appeared to be a better substrate than ribose-5-phosphate (19). That ribulose-5-phosphate is actually the compound which is cleaved to yield triose phosphate, while ribose-5-phosphate serves as acceptor, was established by the demonstration that certain other aldoses can be substituted for ribose-5-phosphate. The following compounds were found to be active as acceptor aldehydes: glycolaldehyde, DL-glyceraldehyde, ribose-5-phosphate, and deoxyribose-5-phosphate. On the other hand D-ribose, acetaldehyde, formaldehyde, and glucose-6-phosphate were inactive under the conditions tested.

Nature of Triose Phosphate Formed from Ribulose-5-phosphate—Isomerase product was incubated with transketolase in the presence of hydrazine as a trapping agent, the hydrazones formed were decomposed with benzaldehyde according to Meyerhof (20), and the triose phosphate assayed with crystalline glyceraldehyde-3-phosphate dehydrogenase in the presence and absence of triosephosphate isomerase. It was found that the predominant triose phosphate was glyceraldehyde-3-phosphate. More directly it was shown that, with crystalline transketolase, glyceraldehyde-3-phosphate dehydrogenase, and arsenate, the addition of isomerase product led to a rapid reduction of DPN, as shown in Fig. 5. It can also be seen in Fig. 5 that the transketolase preparation was free of triosephosphate isomerase, since, in a system containing transketolase, glyceraldehyde-3-phosphate dehydrogenase, and the equilibrium mixture of triose phosphates (21), oxidation of dihydroxyacetone did not take place until triosephosphate isomerase was added. It is evident therefore that glyceraldehyde-3-phosphate is the triose phosphate produced in the splitting of ribulose-5-phosphate by yeast transketolase. This system has been used for the quantitative determination of ribulose-5-phosphate in mixtures containing triose phosphates, HDP, and hydroxypyruvate. The latter compounds interfere with the assay by the glycerophosphate dehydrogenase system, which is not free of aldolase, triosephosphate isomerase, and lactic dehydrogenase.

Hydroxypyruvate As Substrate for Transketolase and Synthesis of Ribulose-5-phosphate—Crystalline transketolase from bakers' yeast catalyzes the decarboxylation of hydroxypyruvate (22) only in the presence of one of the acceptor aldehydes mentioned above. When DL-glyceraldehyde-3-phosphate, or D-glyceraldehyde-3-phosphate, generated from HDP by aldolase, served as acceptor aldehyde, a ketopentose phosphate was formed. The stoichiometric relationship of CO_2 evolution and ketopentose formation is demonstrated in Table II, which also shows the absence of pyruvate carboxylase in these preparations. No cleavage of ribulose-5-phosphate

took place until the acceptor was added, indicating that no ribose-5-phosphate was formed under these conditions. ThPP was required for the decarboxylation of hydroxypyruvate; this could be demonstrated only after dialysis of the enzyme against 0.6 per cent EDTA-0.9 per cent KCl.

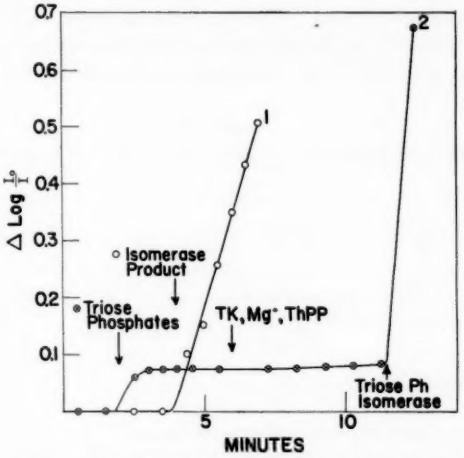


FIG. 5. Demonstration of D-glyceraldehyde-3-phosphate as the triose phosphate formed in the cleavage of ribulose-5-phosphate. The following components were present in both reactions represented by Curves 1 and 2: 0.1 ml. of glycylglycine buffer, 0.5 M, pH 7.6; 0.04 ml. of potassium arsenate, 0.15 M; 0.05 ml. of DPN, 10 mg. per ml. (67 per cent pure); 0.05 ml. of $MgCl_2$, 0.06 M; 0.05 ml. of ThPP, 0.1 per cent; 0.01 ml. of triosephosphate dehydrogenase seven times recrystallized, 3.2 mg. per ml. containing 600 γ of Versene, pH 7.0, per ml.; 265 units of transketolase (specific activity = 150,000) and water to a final volume of 1.0 ml. Curve 1, triosephosphate dehydrogenase was added at zero time; transketolase, Mg^{++}, and ThPP where indicated, and the reaction started by the addition of 0.03 ml. of a 1.5 per cent solution of the ammonium salt of isomerase product. Curve 2, triosephosphate dehydrogenase was added at zero time; 0.02 ml. of a solution of the equilibrium mixture of triose phosphates containing 19 μmoles of total triose phosphate per ml. was added as indicated; transketolase, Mg^{++}, and ThPP were added as indicated, and finally 0.01 ml. of a 10 mg. per ml. solution of triosephosphate isomerase was added.

The ketopentose phosphate synthesized by transketolase from hydroxypyruvate behaved in all respects like ribulose-5-phosphate obtained by decarboxylation of phosphogluconate. It was split by transketolase to yield triose phosphate, but only in the presence of acceptor aldehyde; it gave a positive carbazole reaction; the product of the orcinol reaction had an absorption spectrum identical to that given by ribulose-5-phosphate (23).

XUM

A large scale preparation of ribulose-5-phosphate was carried out as follows: 800,000 units of crystalline transketolase (specific activity 93,000) were incubated with 10 ml. of 0.3 M $MgCl_2$, 5 ml. of 0.5 per cent ThPP, 100 mg. of crystalline aldolase (from rabbit muscle), 15 ml. of 0.2 M HDP, 20 ml. of 0.5 M glycylglycine, pH 7.0, 90 ml. of 0.044 M hydroxypyruvate in a final volume of 210 ml. The reaction mixture was incubated for 2 hours at 30°, after which period the reaction was stopped by the addition of 20 ml. of 50 per cent TCA. As indicated by an orcinol determination,

TABLE II

Decarboxylation of Hydroxypyruvate by Crystalline Transketolase from Bakers' Yeast

Flask No.	Conditions	CO_2 liberated*	Ketopentose phosphate formed
		μmoles	*μmoles*
1	Complete system	2.25	2.8
2	" "	5.7	5.7
3	" "	6.3	6.7
4	No transketolase	<0.1	0.0
5	" HDP	<0.1	0.0
6	Pyruvate instead of hydroxypyruvate	<0.1	0.0

* Corrected for CO_2 retention.

The complete system contained 200 γ of seven times recrystallized aldolase; 500 γ of crystalline transketolase (specific activity = 90,000), 50 μmoles of HDP, 0.6 μmole of ThPP, 30 μmoles of $MgCl_2$, 100 μmoles of glycylglycine buffer, pH 7.2, and potassium hydroxypyruvate as follows: Flasks 1 and 2 contained 15 and 25 μmoles, respectively, and the remaining flasks 50 μmoles. The final volume was adjusted to 2.0 ml. with water. The reaction was allowed to proceed for 60 minutes, at which time an equal volume of 10 per cent trichloroacetic acid was added. The neutralized filtrates were assayed for ribulose phosphate with crystalline transketolase, glyceraldehyde-3-phosphate dehydrogenase, DPN, and arsenate in the presence of excess ribose-5-phosphate as acceptor.

approximately 900 μmoles of pentose phosphate were synthesized. The precipitated protein was removed by centrifugation, the pH was then adjusted to 6.9, and the barium-soluble-alcohol-insoluble fraction was collected. This salt, amounting to 3.0 gm., was thoroughly extracted with small portions of water until no more pentose was eluted; the combined washings were then reprecipitated at pH 6.9 with alcohol. The precipitate (1.8 gm.) was eluted with water as above; 690 μmoles of pentose phosphate (as shown by the orcinol reaction) were obtained in a final volume of 32 ml. The Ba^{++} was then removed with ammonium sulfate, and the solution placed on a Dowex 1 formate column 5 $cm.^2 \times 30$ cm. The column was washed with water and the pentose phosphate eluted with 0.1 M formic

acid-0.03 M Na formate, pH 3.1 (23), and 25 ml. fractions were collected with the aid of an automatic fraction collector. The pentose phosphate was eluted between 2700 and 3600 ml. of eluent. The fractions containing pentose phosphate (500 μmoles) were pooled and the pentose phosphate was precipitated as the barium salt at pH 6.5.

Formation of Heptulose Phosphate by Crystalline Transketolase—As shown above, transketolase catalyzes the formation of glyceraldehyde-3-phosphate from ribulose-5-phosphate only in the presence of a suitable acceptor alde-

TABLE III

Synthesis of Heptulose Phosphate by Crystalline Transketolase

Tube No.	Components		Heptulose phosphate synthesized
			μmoles
1	Ribose-5-phosphate		0.05
2	"	+ hydroxypyruvate	2.5
3	"	+ 1-erythrulose	3.2
4	"	+ F-6-P	1.1
5	Ribulose-5-phosphate		0.25
6	Ribose-5-phosphate	+ ribulose-5-P	3.1

All the tubes contained 100 μmoles of glycylglycine buffer, pH 7.5; 15 μmoles of $MgCl_2$; 100 γ of ThPP; 2200 units of crystalline transketolase (specific activity = 140,000); 6.0 μmoles of synthetic ribose-5-phosphate (except Tube 5). Where indicated, the following components were added: 4.8 μmoles of hydroxypyruvate (potassium salt); 20 μmoles of L-erythrulose; 20 μmoles of fructose-6-phosphate (F-6-P); 3.3 μmoles of ribulose-5-phosphate (from hydroxypyruvate; isolated and purified on Dowex 1 formate, as described in the text). The tubes were incubated for 30 minutes at 38° and analyzed for heptose formation by the cysteine reaction of Dische (13). 24 hours after addition of cysteine, the tubes were read at 510 mμ. Zero time samples with and without internal sedoheptulosan standard were analyzed for each reaction mixture and appropriate corrections made.

hyde. With ribose-5-phosphate as acceptor, the products of the reaction should be triose phosphate and a heptulose phosphate. The data in Table III indicate that this is indeed the case. Heptulose formation was determined by the method of Dische (13) with sedoheptulosan as a standard. As can be seen in Table III, heptulose synthesis with crystalline transketolase is observed only when both ribose-5-phosphate and a source of an active 2-carbon fragment are present. That a transfer of a 2-carbon fragment from ribulose-5-phosphate to ribose-5-phosphate is involved is indicated by the demonstration of heptulose phosphate synthesis from hydroxypyruvate in the presence of ribose-5-phosphate.

The product of the reaction of ribose-5-phosphate with hydroxypyruvate catalyzed by crystalline transketolase has been isolated as a barium salt

and purified by means of chromatography on Dowex 1 formate. Elution of the heptulose phosphate was followed by the cysteine reaction (13). Its chromatographic behavior on this resin was identical with that reported by Horecker *et al.* (19) for sedoheptulose-7-phosphate in its early elution and in its remarkably sharp elution curve. The eluted heptulose phosphate was again isolated as a barium salt. Paper chromatographic comparison

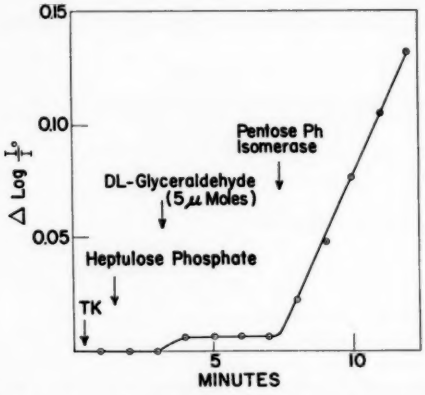


FIG. 6. Formation of triose phosphate from heptulose phosphate by transketolase in the presence of acceptor aldehyde and pentosephosphate isomerase. The following components were present in the reaction mixture: 0.05 ml. of glycylglycine buffer, 0.5 M, pH 7.6; 0.05 ml. of DPNH, 0.0014 M; 0.05 ml. of a 1 per cent solution of a rabbit muscle fraction containing glycerophosphate dehydrogenase and triosephosphate isomerase; 0.1 ml. of $MgCl_2$, 0.3 M; 0.02 ml. of ThPP, 5 per cent; 0.05 ml. of heptulose phosphate, 17 μmoles per ml.; 0.02 ml. of transketolase, 110,000 units per ml. (specific activity = 16,000); 0.02 ml. of DL-glyceraldehyde, 0.25 M; 0.03 ml. of yeast pentosephosphate isomerase diluted 1:10 in water and prepared as described in the text; and water to a final volume of 1.0 ml. All the components except those indicated in the figure were added before the reaction was started by the addition of transketolase.

of this heptulose phosphate in various solvents (80 per cent ethanol-2 per cent acetic acid; 80 per cent ethanol-0.6 per cent boric acid; 80 per cent ethanol) with a sample of sedoheptulose-7-phosphate obtained from Dr. B. L. Horecker indicated identical behavior of these two compounds. The spectra obtained in the cysteine reaction and in the orcinol reaction, and the color reaction on paper as described by Benevue and Williams (24), were identical to those obtained with sedoheptulose.

Fructose-6-phosphate As Substrate for Transketolase—It has been reported (25) that transketolase will transfer the ketol group from fructose-6-phosphate to ribose-5-phosphate, yielding heptulose phosphate, and to

glyceraldehyde-3-phosphate, yielding ribulose-5-phosphate. Heptulose phosphate synthesis from fructose-6-phosphate and other donors of active aldehyde is shown in Table III. The other products of these reactions are under investigation. The compound formed from fructose-6-phosphate is presumably a tetrose-4-phosphate. From the reaction mixture a barium salt was isolated which was oxidized by glyceraldehyde-3-phosphate dehydrogenase in the presence of DPN and arsenate. The amount of heptulose phosphate synthesized could be considerably increased when both HDP and aldolase were added. This is in accord with the observations of Horecker, who showed that erythrose and dihydroxyacetone phosphate are condensed by aldolase to give sedoheptulose-1-phosphate, and who isolated a heptulose diphosphate from a system which was expected to form triose phosphate and tetrose phosphate (26).

Reversibility of Transketolase-Catalyzed Reaction—In the presence of transketolase and an acceptor aldehyde such as DL-glyceraldehyde, heptulose phosphate (obtained from the reaction between hydroxypyruvate and ribose-5-phosphate) yielded triose phosphate only upon addition of pentose-phosphate isomerase, as seen in Fig. 6. It is therefore apparent that the cleavage of heptulose phosphate yielded ribose-5-phosphate which was isomerized to ribulose-5-phosphate. The latter was then split to glyceraldehyde-3-phosphate in the presence of the acceptor aldehyde.

Formation of Hexose Monophosphate—Partially purified preparations of yeast transketolase were found to give rise to glucose-6-phosphate in the presence of isomerase product (27). Crystalline preparations of transketolase alone did not form hexose monophosphate, but on addition of highly purified transaldolase[3] fructose-6-phosphate accumulated. The latter was measured with glucose-6-phosphate dehydrogenase in the presence of rabbit muscle hexosephosphate isomerase. The reaction did not proceed unless transketolase was added, thus demonstrating the complete separation of transaldolase from transketolase.

DISCUSSION

The formation of glyceraldehyde-3-phosphate from ribose-5-phosphate in partially purified preparations from yeast has been demonstrated to proceed in two steps catalyzed by separable enzymes. In the first step an equilibrium mixture of ribose-5-phosphate and ribulose-5-phosphate (isomerase product) is formed.

(1) Ribose-5-phosphate $\rightleftharpoons$ ribulose-5-phosphate

In the second step ribulose-5-phosphate and ribose-5-phosphate are

[3] The experiments with highly purified transaldolase preparations were carried out in collaboration with Dr. P. A. Srere.

transformed by transketolase into a mixture of heptulose-7-phosphate and glyceraldehyde-3-phosphate.

(2) Ribose-5-phosphate + ribulose-5-phosphate

$\rightleftharpoons$ heptulose-7-phosphate + glyceraldehyde-3-phosphate

It is not claimed that the above formulation of the reaction is operative in systems other than yeast. Whether it applies also to liver and spinach preparations (19) cannot be stated until a separation of the two steps has been accomplished in these systems. Since the spinach enzyme has been highly purified (19) without effecting a separation of the two activities, the possibility of the existence of a "double headed enzyme" containing both transketolase and pentosephosphate isomerase may be considered. Such a mechanism would have the particular advantage of a greater efficiency in heptulose synthesis, a reaction which may play an important rôle in photosynthesis (28).

It has been suggested (4) that, in the presence of a keto sugar or hydroxypyruvate, transketolase catalyzes the formation of "active aldehyde," which then condenses with an "acceptor aldehyde" to form a new keto sugar, as given in Reaction 2. Although it is possible to demonstrate an absolute requirement for the "acceptor aldehyde," it should be pointed out that this does not represent evidence for the formation of "active glycolaldehyde." As an alternative explanation for the above findings, a displacement of the triose phosphate moiety of ribulose-5-phosphate by the acceptor aldose might be considered. Such a mechanism could take place without the intermediate formation of an "active aldehyde." Experiments which should differentiate between the two alternative hypotheses are in progress.

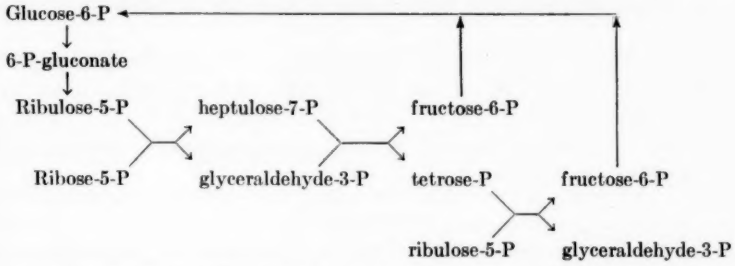
A third enzymatic step which is required for glucose-6-phosphate formation from pentose phosphate has been demonstrated. The reaction has been suggested to consist of a transaldolization (1) and the enzyme has been partially purified (26, 27).

(3) Heptulose-7-phosphate + glyceraldehyde-3-phosphate

$\rightleftharpoons$ fructose-6-phosphate + "tetrose-4-phosphate"

Since transketolase has been found to cleave fructose-6-phosphate (25), a scheme has been proposed (2) for a complete cycle of glucose oxidation with the participation of six enzymes (glucose-6-phosphate, phosphogluconate dehydrogenase, transketolase, transaldolase, hexose phosphate, pentosephosphate isomerase). Many features of this scheme have also been advanced by Horecker (3). This formulation of the cycle is consistent with quantitative data on the yield of hexose monophosphate from ribose-5-phosphate (1) and also with recent isotope studies on the redistri-

bution of labeled glucose and ribose phosphates (3). The significance and further details of the cycle have been discussed elsewhere (2).



It should be stressed that the specificity of yeast transketolase has not as yet been fully explored because only a few phosphorylated keto sugars are available. A unique configuration about the asymmetric carbon atom at the point of cleavage does not seem to be essential for transketolase action, as evidenced by the cleavage of fructose-6-phosphate. This suggests the possibility that other keto sugars such as xylulose-5-phosphate and alloheptulose-7-phosphate may be cleaved and synthesized by yeast transketolase.

The significance of the transketolase-catalyzed condensation of triose phosphate and "active glycolaldehyde" for the biosynthesis of pentoses remains to be determined. Glyceraldehyde-3-phosphate is formed during glycolysis, while "active glycolaldehyde" may be derived from serine via hydroxypyruvate (29) or by cleavage of fructose-6-phosphate as described in this paper. On the other hand, the enzymes and substrates required for ribulose-5-phosphate formation by oxidative decarboxylation of glucose-6-phosphate via 6-phosphogluconate are also available to many cells. Attempts have been made (30, 31) to evaluate the significance of the glucose-6-phosphate shunt pathway in various tissues by measuring radioactive CO_2 derived from C-1- and C-6-labeled glucose. The relative rôle of the 2- and 3-carbon condensation which was first indicated by isotope experiments (32) is even more difficult to evaluate quantitatively because of the possible occurrence of exchange reactions.

SUMMARY

1. The purification and crystallization of transketolase from bakers' yeast have been described and its requirement for thiamine pyrophosphate and magnesium ions demonstrated.

2. In the presence of the crystalline enzyme, several keto sugars as well as hydroxypyruvate give rise to an "active glycolaldehyde," which is condensed with an "acceptor aldehyde" to give rise to a new keto sugar. With ribulose-5-phosphate as donor and ribose-5-phosphate as acceptor,

a heptulose phosphate and a triose phosphate are formed. The triose phosphate was identified as D-glyceraldehyde-3-phosphate.

3. Fructose-6-phosphate is shown to be a donor of "active glycolaldehyde."

4. The formation of fructose-6-phosphate from pentose phosphate is shown to be dependent on the participation of both transketolase and transaldolase.

5. Based on the above finding, a glucose-6-phosphate oxidation cycle catalyzed by six enzymes is postulated.

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COMPARISON OF THE METABOLISM OF C^{14}-LABELED LACTOSE, GLUCOSE, AND GALACTOSE IN RATS

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The importance of milk in human nutrition makes it desirable to understand better the metabolism of lactose. Much of the experimental work on the utilization of lactose has been conducted with rats (8, 16). Other investigators have employed pigs, chickens, dogs, and calves (10–12, 19), while infants, children, and adults were used under suitable conditions (2, 14, 21, 23). We wish to report here observations dealing with the fate of the carbon of administered C^{14}-labeled lactose, glucose, and galactose in rats. Glucose and galactose were included in this study in order to facilitate the evaluation of the metabolic fate of lactose. The yields of C^{14} in the expired air, liver, feces, urine, and carcass served as a measure of the extent of sugar utilized after the radioactive sugars were introduced into the rat by stomach tube and injection into the caudal vein.

EXPERIMENTAL

Isotopic Compounds[1]—4 to 6 mg. each of D-glucose-U-C^{14} (Oak Ridge National Laboratory and The Atomic Center, Inc.), D-glucose-1-C^{14}, D-galactose-1-C^{14}, and D-lactose-1-C^{14} (National Bureau of Standards) were dissolved in 1 to 2 ml. of physiological saline solution and in 5 ml. of water for intravenous and oral administration, respectively. In those experiments in which 100 to 250 mg. of the sugar were given orally, 4 to 6 mg. of the radioactive sugar were diluted gravimetrically with non-isotopic sugar and dissolved in 5 ml. of water.

In order to establish the radiochemical homogeneity of the radiosugars, they were subjected to descending one-dimensional paper chromatography (15) and radioautography with Eastman Kodak "no screen" x-ray film. The length of exposure of the paper chromatograms to the x-ray film was dependent upon the amount of activity present on the paper chromatogram.

In the case of galactose-1-C^{14} and glucose-U-C^{14}, the presence of only one detectable spot on each of the radioautograms indicated that galactose and glucose were free of contamination by any radioactive impurities.

Radioautograms of glucose-1-C^{14} revealed radioactive impurities having

[1] Glucose uniformly labeled in all carbon positions with C^{14} is referred to as glucose-U-C^{14}.

R_G[2] values of 0.08, 0.12, 0.20, and 0.30. The purification of this sugar was accomplished by column chromatography by the charcoal-Celite technique described by Whistler and Durso (24) with water as the eluting solvent.

Chromatographic and radioautographic analysis of one batch of lactose-

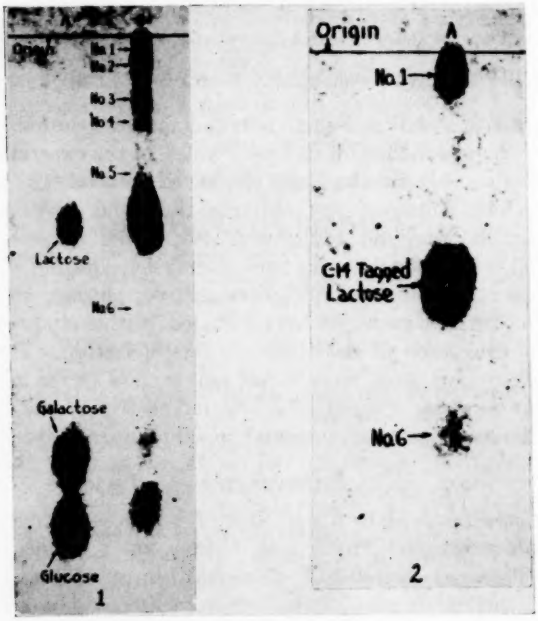

FIG. 1. One-dimensional paper chromatogram of a sample of lactose-1-C^{14} (0.98 μc. per mg.) showing the presence of substances, other than lactose, which give a positive color reaction with ammoniacal silver nitrate. *A*, lactose, galactose, glucose standards; *B*, 157.6 γ of the sample of lactose-1-C^{14} applied in portions of 3.94 γ per 2 μl. (forty 2 μl. applications).

FIG. 2. A radioautogram (72 hours exposure) of the sample of lactose-1-C^{14} depicted in Fig. 1. *A*, 39.4 γ (0.0386 μc. activity) of the sample applied in 3.94 γ per 2 μl. portions (ten 2 μl. applications).

1-C^{14} (0.98 μc. per mg.) revealed the presence of six additional substances (Fig. 1), of which two were radioactive (Fig. 2). Purification of this lactose was achieved by the column chromatographic technique of Whistler and Durso. Scanning analysis[3] (Fig. 3) showed the purified lactose to be

[2] R_G is the ratio of the distance moved by the spot to the distance moved by glucose on the same paper.

[3] Courtesy of Dr. R. C. Anderson, Brookhaven National Laboratory.

suitable for metabolic studies. A second sample of lactose-1-C^{14} (1.26 μc. per mg.) was analyzed by similar techniques and was found to be sufficiently pure (Fig. 4) for use in these studies.

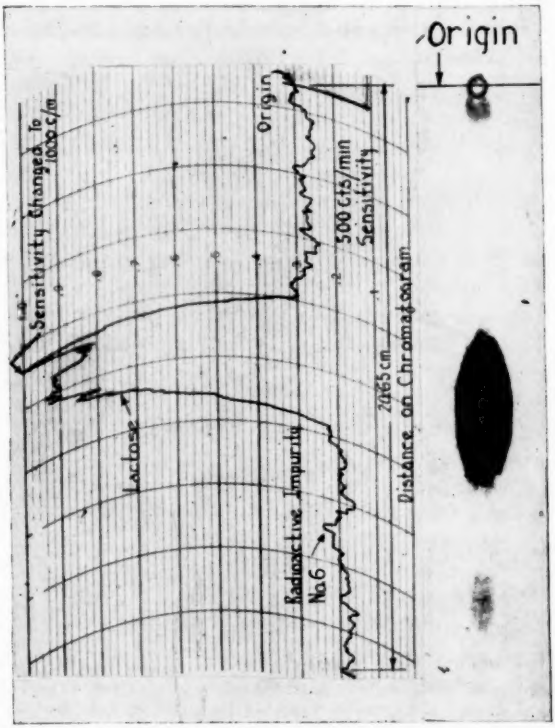

FIG. 3. Curve obtained by scanning a chromatogram of a portion of the lactose depicted in Figs. 1 and 2 after purification by column chromatography. The curve showed that the radioactive impurities revealed on the radioautogram (2 weeks exposure, shown at the right of the curve) were insignificant. The chromatogram used for the radioautogram and scanner contained a total of 0.038 μc. of lactose. A distance of 1 cm. traversed on the recorder was equal to 1.18 cm. on the chromatogram.

Administration of Dose—Forty male rats (Sprague-Dawley strain), weighing from 180 to 200 gm., were given doses of the labeled sugars; sixteen animals received injections into the caudal vein and twenty-four by stomach tube. All rats were maintained on a sugar-free diet offered *ad libitum* prior to and during the experiment. The experiments varied as to duration, as shown in Tables I to IV. Those animals which received

doses by stomach tube were fasted for 18 hours prior to the administering of the radioactive sugars. The diet contained 24 per cent casein, 4 per cent Phillips and Hart salt mixture (18), 6 per cent butter fat, 2 per cent cod liver oil, 2 per cent liver concentrate powder, and 62 per cent cornstarch. To this diet were added vitamins E, K, and B complex.

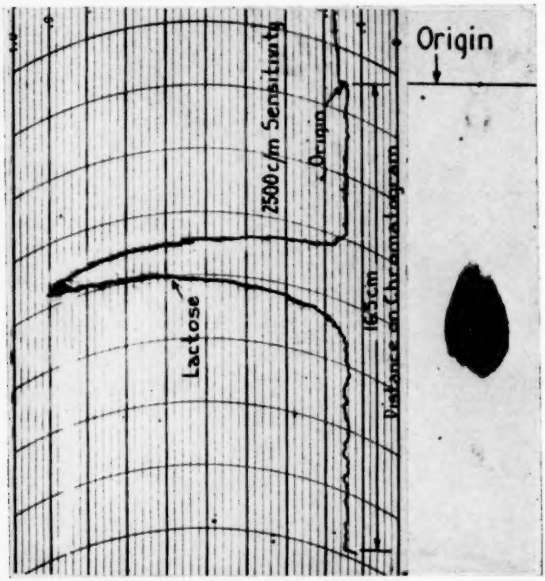

FIG. 4. Curve resulting when a second sample of lactose-1-C^{14} (1.26 μc. per mg.) obtained from the National Bureau of Standards was scanned. The curve showed that the two radioactive impurities revealed by the radioautogram (2 weeks exposure) were insignificant. The chromatogram which was radioautographed (shown at the right of the curve) and scanned contained 0.063 μc. of lactose. A distance of 1 cm. traversed on the recorder was equal to 1.18 cm. on the chromatogram.

Collections and Assays—Following the dosing, the rats were housed in metabolism cages which were, in turn, placed in respiration chambers. Air was drawn through the chamber at a rate necessary to maintain 2 cm. of water pressure. Carbon dioxide in the respired air was collected at specified intervals by drawing the air through an absorption tower containing 2.5 N NaOH. A porous glass disk at the bottom of the tower served to break the stream of air into fine bubbles. Urine was collected at 24 hour intervals. At the termination of the respired air collections, the respiration chamber was opened and the animal was sacrificed by chloroform inhalation. At this time, feces were collected for radioassay. In

two cases (glucose and lactose), blood was taken by intracardial puncture prior to the sacrifice of the animals. Livers were excised only from those animals that received lactose and glucose via stomach tube. Carcasses were autoclaved for 1 hour at 15 pounds pressure and converted into a uniform paste in a Waring blendor prior to radioassay.

Respired air samples were assayed by the method of Zilversmit *et al.* (26). The apparatus described by Anderson *et al.* (1) was used in the dry combustion procedure. In the wet combustion procedure reagents described by Van Slyke *et al.* (22) and the apparatus of Steele and Sfortunato (20), modified by Anderson, were employed.[4] The carbon dioxide from the combustion procedures was precipitated as $BaCO_3$, collected on filter paper, dried, weighed, and counted in a windowless gas flow detector (Nuclear Instrument and Chemical Corporation, model 1 D-46A). Sufficient counts were made to reduce the counting error to less than ± 3 per cent; the counts were corrected for coincidence losses, background, and self-absorption.

RESULTS AND DISCUSSION

Comparison of Metabolism of Sugars Following Intravenous Administration—The rates at which C^{14} appeared as respiratory CO_2 are presented in Figs. 5, 6, and 7 and are expressed as the per cent per hour of the administered radiocarbon recovered *versus* the time following the administration of the isotope. No significant differences exist in the rate at which the injected labeled sugars were oxidized by animals within each experimental group. During the 1st hour there was a lag in $C^{14}O_2$ output. However, the rate accelerated, reached its maximum during the succeeding 2 to 4 hours, and thereafter diminished progressively with time.

Glucose-U-C^{14}, glucose-1-C^{14}, and galactose-1-C^{14} exhibited similar catabolic patterns, as shown by the recovery of radioactive CO_2 in the respired air (Tables I to IV). The oxidation of both uniformly labeled glucose and glucose-1-C^{14} was very rapid, as shown by the large recoveries of activity (64 per cent) in the respired air within the first several hours (Tables I and II). Only an additional 16 per cent of the administered activity was detected in the respired air in the 4 days following the initial oxidation of the uniformly labeled sugar. The conversion of glucose to CO_2 via the Embden-Meyerhof glycolytic pathway (3) may explain the small differences in the respired air recoveries in the experiments in which uniformly labeled glucose and glucose-1-C^{14} were used. Since the sequence in which the several carbon atoms of glucose appeared as CO_2 in a respiring system according to the Embden-Meyerhof pathway is 3,4, 2,5, and 1,6, within a given period, the yields of $C^{14}O_2$ from glucose-U-C^{14}

[4] Dr. R. C. Anderson, private communication.

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catabolism are slightly greater than those from glucose-1-C^{14} catabolism (Tables I and II). The lower rate of $C^{14}O_2$ oxidation with galactose is probably attributable to the difference in the steric configuration about the 4 carbon. Nevertheless, 61 per cent of the galactose at the 6 mg. level was oxidized within 24 hours (Table III).

TABLE I

Conversion of Glucose-U-C^{14} to $C^{14}O_2$ by Normal Rats

Cumulative radiochemical yield in expired air from glucose-U-C^{14}.

Time elapsed	Caudal vein injection			Stomach tube		
	5 mg.	5 mg.	5 mg.	2.5 mg.	4 mg.	250 mg.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	4.90	5.36	1.11			
2				11.78		25.02
3.5	39.59	30.93	44.19			
4				19.40		42.46
7				26.72		48.78
8	61.47	61.61	68.27			
13.5	66.07	66.32	74.16			
24	69.47	69.76	77.31	59.95		64.09
31	71.21	71.66	78.83	61.62		65.82
48	73.67	74.58	81.55	67.03		68.36
72	76.09	77.58	84.77			
102	77.10	78.35	85.30			
	Recovery of C^{14} in urine					
24	4.52	4.53	4.91		1.26	1.39
48	5.21	5.02	5.18	2.13	1.60	1.66
72	5.43	5.22	5.38			
102	6.19	5.89	5.76			
	Recovery of C^{14} in feces					
	0.97	1.08	0.42	0.94	1.07	1.35
	Recovery of C^{14} in liver					
				7.19	1.46	1.39
	Recovery of C^{14} in carcass					
	15.36	10.42	13.01	22.24	20.60	21.21
Total....	99.62	95.74	104.49	99.53		93.97

7 per cent of the administered lactose activity appeared in the respired air within a period of 48 hours (Table IV). The occurrence of small quantities of the enzyme, lactase, in the liver and kidneys (17) suggests the mechanism for the cleavage of the 1,4 linkage of lactose and the subsequent oxidation of the labeled glucose moiety.

TABLE II

Conversion of Glucose-1-C^{14} to $C^{14}O_2$ by Normal Rats

Cumulative radiochemical yield in expired air from glucose-1-C^{14}.

Time elapsed	Caudal vein injection		Stomach tube	
	4 mg.	5 mg.	5 mg.	250 mg.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*
2	42.03	45.98	13.08	17.14
4			16.68	24.25
6	55.14	59.23	21.47	33.12
23	66.05	71.87	34.93	48.06
27			36.28	48.83
30	67.88	72.84	37.39	49.45
48	70.67	75.22	46.63	51.86
55			48.69	52.29
72			50.76	53.31
	Recovery of C^{14} in urine			
24			1.33	2.07
48	3.69	3.54		
72			1.64	2.95
	Recovery of C^{14} in blood			
72			0.17	0.13
	Recovery of C^{14} in feces			
	2.16	0.87	0.92	1.22
	Recovery of C^{14} in liver			
			5.70	1.26
	Recovery of C^{14} in carcass			
	23.42	18.92	40.80	39.77
Total	99.94	98.55	99.99	98.64

Small quantities of radioactivity were recovered in the urine with the exception of the urine of those rats receiving lactose. Twice as much

TABLE III

Conversion of Galactose-1-C^{14} to $C^{14}O_2$ by Normal Rats

Cumulative radiochemical yield in expired air from galactose-1-C^{14}.

Time elapsed	Caudal vein injection			Stomach tube				
	6 mg.	6 mg.	6 mg.	6 mg.	100 mg.	250 mg.	250 mg.	250 mg.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent,*	*per cent*	*per cent*
1				6.94	1.04	0.39	0.68	0.33
2	30.10	30.38	33.01	19.34	2.92	2.11	1.00	1.62
3				25.70	4.11	3.87	4.47	4.44
4				29.37	4.97	6.54	8.10	9.06
5				32.91	5.46	8.01	12.49	12.59
7	50.50	53.15	56.14	35.84	6.03	8.99	18.71	17.49
24	56.32	60.77	64.79	52.24	14.04	15.58	29.35	31.26
27	56.98	61.29	65.31	53.28	16.64	16.24	30.05	32.86
31	57.82	62.12	66.18	54.64	16.95	17.39	32.11	34.79
48	60.05	64.37	67.41	60.35	24.15	23.18	35.83	39.25
55	60.93	65.17	68.59		25.40	28.83	36.83	40.74
72	62.31	66.16	70.21		29.16	35.03	39.34	41.00
79	62.58	66.77	70.71		30.05	36.19	39.96	42.11
103	64.39	68.09	72.98		32.62	38.87		
168	67.48	72.01	77.08		39.98	44.86		
240	68.73	73.45	78.59					
	Recovery of C^{14} in urine							
24	8.65	4.27	10.42	5.22	11.27	30.27	33.09	22.18
48	9.56	5.09	11.52	5.59	11.83	31.99	33.93	23.06
72	10.17	5.71	12.49		12.26	33.12	35.86	23.58
168					13.18	34.79		
240	11.07	7.05	14.14					
	Recovery of C^{14} in feces							
	1.29	2.93	2.17	1.50	1.27	0.97	0.29	0.83
	Recovery of C^{14} in carcass							
	9.50	6.01	7.98	30.17	32.04	19.89	23.55	33.41
Total..	90.59	89.44	102.88	97.61	86.47	100.51	99.66	99.93

activity appeared in the urine of rats given galactose (10 per cent) as in the urine of rats given glucose (5 per cent), which is in agreement with the

TABLE IV

Conversion of Lactose-1-C^{14} and Glucose-1-C^{14} + Galactose-1-C^{14} to $C^{14}O_2$ by Normal Rats

Cumulative radiochemical yield in expired air from lactose-1-C^{14} and glucose-1-C^{14} + galactose-1-C^{14}.

Time elapsed	Caudal vein injection		Stomach tube			
	5 mg. lactose-1-C^{14}	5 mg. lactose-1-C^{14}	2.8 mg. lactose-1-C^{14}	250 mg. lactose-1-C^{14}	3.5 mg. glucose-1-C^{14} + 3.5 mg. galactose-1-C^{14}	60 mg. glucose-1-C^{14} + 60 mg. galactose-1-C^{14}
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1					5.66	6.35
2	0.84	0.55	7.24	10.65	14.58	12.62
4			13.51	19.11		
5			16.78	23.56	19.96	16.30
7	2.65	3.49	19.08	28.27	23.44	19.30
24	3.96	6.63	30.47	35.43	33.32	24.37
31	4.47	7.03	32.16	38.26	35.72	28.31
48	5.24	8.01	38.01	44.51	42.47	35.53
55			39.19	45.75		
72			41.58	49.20		
	Recovery of C^{14} in urine					
24	76.50	82.91	1.00	2.66	3.25	11.40
48	78.98	86.08			4.02	12.42
72			1.91	3.77		
	Recovery of C^{14} in feces					
	0.07	0.24	4.48	4.37	1.71	1.23
	Recovery of C^{14} in liver					
			8.25	12.79	33.56	28.86
	Recovery of C^{14} in blood					
			0.53	0.14		
	Recovery of C^{14} in carcass					
	14.73	4.78	42.17	35.23	17.17	21.06
Total.....	99.02	99.11	98.92	105.50	98.93	99.10

work of Cori and Cori extrapolated to low levels (5, 6). Radioactivity from lactose appeared almost entirely in the urine (83 per cent). Radioautograms showed the presence of lactose and three additional components, indicating that the majority of C^{14} activity in the urine was represented by unchanged lactose, while a small portion of the activity was present in the unidentified components.

The radioactivity (0.2 to 2 per cent) appearing in the feces did not suggest reabsorption via the intestine. It did appear, however, that the feces were contaminated by the urine, since the cage construction did not insure the absence of contact between urine and feces, and therefore it seemed logical to consider these data obtained from additional recovery in the urine.

The differences in carcass retention among the four sugars varied from 7 to 21 per cent (Tables I to IV). Since the 1,6 carbons of glucose would be oxidized to CO_2 last in the Embden-Meyerhof sequence, the retention of C^{14} activity would be greater with glucose-1-C^{14}, as all the radioactivity appears solely in the 1st carbon of the hexose. The retention of approximately 8 per cent radioactivity in the carcass from the galactose administration is in agreement with the data, as it represents approximately a 5 per cent difference from the activity recovered in the carcass and urine after giving glucose-1-C^{14}. Lactose retention was rather high (10 per cent).

Comparison of Metabolism of Sugars Following Oral Administration—As with the intravenous administrations, uniformly labeled glucose was catabolized to CO_2 to a greater extent than glucose-1-C^{14}, galactose, lactose, and an equal mixture of the 1-C^{14}-labeled glucose and galactose (Tables I to IV). With lactose, doses of both 2.8 and 250 mg. exhibited similar patterns of CO_2 catabolism, and most of the C^{14} oxidation occurred within the first several hours (Fig. 7, Table IV). Assuming that the enzymatic hydrolysis of the sugar occurred in the intestine, the subsequent mechanisms that occurred represented only the metabolism of the glucose moiety. This follows since the metabolic pictures of lactose-1-C^{14} and glucose-1-C^{14} were almost identical (Tables II and IV). The quantity of radioactivity recovered in the respired air within 24 hours after lactose administration was 30 to 35 per cent, which was approximately equal to that of both the dosages at lower levels of glucose-1-C^{14} (35 per cent) and the labeled sugar mixture (33 per cent). There was a considerable difference (16 per cent) in the quantity of radioactivity appearing in the respired air from glucose-U-C^{14} compared with that from glucose-1-C^{14}. This difference can be attributed to the Embden-Meyerhof glycolytic scheme, as previously explained. At the 6 mg. level of galactose, the $C^{14}O_2$ catabolism was similar to that of the uniformly labeled glucose oxidation. With increasing concentrations of galactose, the C^{14} oxidation was greatly

reduced (approximately 30 per cent recovered in 24 hours) compared with that at the 6 mg. level. Although the data from the administration of equal quantities of glucose-1-C^{14} and galactose-1-C^{14} showed a marked decrease (7 to 15 per cent) in the respiratory activity, the over-all metabolic

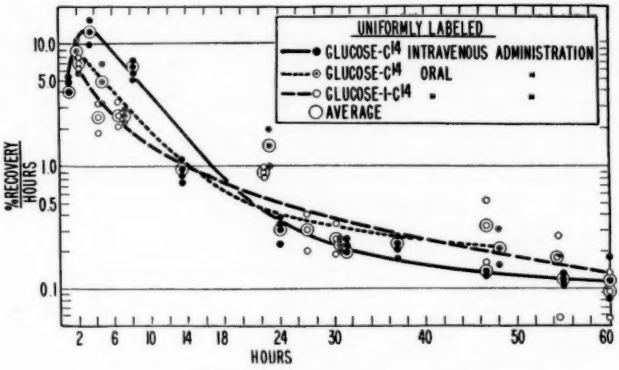

FIG. 5. Radiochemical yields of expired $C^{14}O_2$

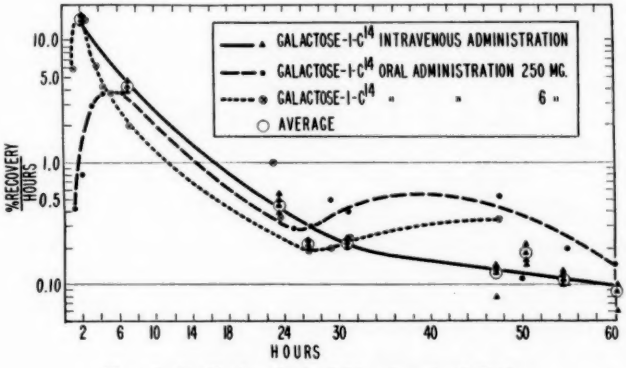

FIG. 6. Radiochemical yields of expired $C^{14}O_2$

picture revealed better utilization of the sugars than when the sugars were administered individually. This is partially attributed to the effect of glucose in increasing tolerance for galactose by absorptive interrelationships and possibly to a more intimate relationship between the two hexoses (4, 7, 13). The differences in rates were small (Figs. 5, 6, and 7); the majority of the radioactivity appeared in the respired air within the first 2 hours in the case of glucose, galactose, and the mixture, whereas the maximal level

of radioactivity was attained 2 to 4 hours later with lactose. Enzymatic hydrolysis in the intestine may account for this time lag. Recovery of radioactivity in the urine was approximately 2 per cent, except after galactose and sugar mixture administrations. At the three levels of galactose, there were large differences in the elimination of C^{14} activity. The recoveries, ranging from 5 to 33 per cent, were proportional to the quantity of sugar administered and were in agreement with the data extrapolated to the low levels reported by Cori and Cori (5, 6). Most of the radioactivity recovered in the urine (Table IV) appeared within the first 24 hours. With the glucose-galactose mixture, the quantity of C^{14} activity in urine was similar to that at the 6 and 100 mg. levels of the administered galactose.

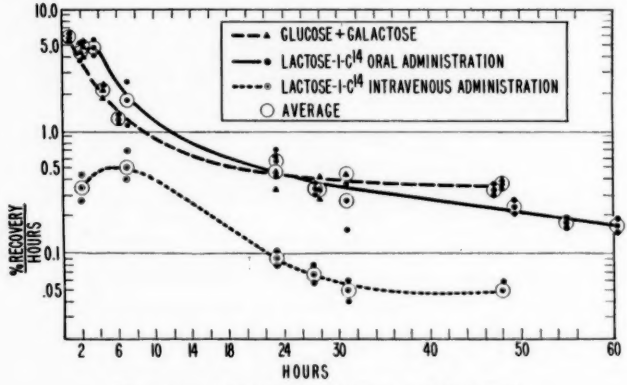

FIG. 7. Radiochemical yields of expired $C^{14}O_2$

In the case of glucose and galactose, radioactivity recovered in the feces was approximately 1 per cent. The data suggested that the activity in the feces possibly was due to unabsorbed sugar and partly to contamination by urine. In regard to lactose, the activity was represented partly by undigested lactose and unidentified substances.

The retention of the sugars or their metabolites in the carcass varied from 17 to 41 per cent in all experiments. Activities from the carcasses of animals receiving lactose-1-C^{14} and glucose-1-C^{14} were almost identical (35 to 42 per cent). With the glucose-galactose mixture, the yield in the carcass was 17 to 21 per cent.

The differences in liver radioactivity following singly and uniformly labeled glucose were almost negligible. With the smaller doses, a larger percentage (5 to 7 per cent) was retained in the liver. The radioactivity was probably in the form of liver glycogen. During the fasting period, the animals presumably lost most of their liver glycogen. Consequently, the

activity recovered in the liver following the dosing must have been the result of glycogen, synthesized proportionately, from the ingested sugar. Increased storage of radioactivity was observed in the liver with lactose administration (8 to 13 per cent). Radioactivity in the liver after the administration of the glucose-galactose mixture ranged from 29 to 34 per cent, almost 3 times the radioactivity recovered after lactose administration. This increase in activity may be in part due to the replacement of glycogen following the fasting period and partially as a result of a more intimate relationship between these sugars.

There was almost no activity in the blood at the end of 72 hours when glucose was administered. The turnover time for glucose is short, and considerable light is shed on the disappearance of glucose from the blood by Feller *et al.* (9), Wicks *et al.* (25), and Zilversmit *et al.* (26).

SUMMARY

Lactose, glucose, and galactose containing C^{14} were administered orally and intravenously to rats. By observing the respiration, retention, and excretion of the radioactive carbon, the rates and manner in which these materials were metabolized were determined.

In the experiments in which the radiocompounds were administered orally, glucose was catabolized to CO_2 to a greater extent than galactose, lactose, or an equal mixture of glucose and galactose. Lactose and the glucose-galactose mixture were retained in the carcass to a greater extent than glucose or galactose administered individually. Galactose excretion via the kidneys was proportional to the dose administered.

With intravenous administration, glucose and galactose were quickly catabolized to respired air. Lactose was excreted primarily by way of the kidneys, and a small percentage of the administered radioactivity appeared in the respired air.

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EFFECTS OF THYROXINE AND SOME RELATED COMPOUNDS ON BACTERIAL OXIDATIONS*

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It was recently observed in our laboratory that the oxidation of cholesterol *in vitro* by intestinal bacteria is stimulated by 2,4-dinitrophenol (1). In preliminary experiments with the same bacterial system, L-thyroxine was also found to accelerate the oxidation of cholesterol and of glucose (2). The work to be described here includes an extension of these studies and, in addition, results obtained with several related compounds.

EXPERIMENTAL

Cells of *Aerobacter aerogenes* were harvested after 40 hours growth at 32° on a half strength nutrient agar medium containing 0.1 mg. per ml. of cholesterol (1). The cells were washed twice in 0.025 M phosphate buffer, pH 7.0, and resuspended in the same buffer at a concentration of 1.3 to 1.5 mg. dry weight of cells per ml. of suspension for use in the Warburg apparatus.

The effects of L-thyroxine,[1] D-thyroxine,[1] DL-thyroxine,[2] L-triiodothyronine,[1] and deaminothyroxine ((3′,5′-diiodophenoxy)-3,5-diiodophenylpropionic acid)[1] on the oxygen consumption of these cells were measured by conventional manometric techniques. These compounds were dissolved by bringing the solution to pH 10.5 with NaOH. The small amount of alkali used did not cause an increase in pH greater than 0.1 pH unit in the

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[1] The authors are much obliged to the Travenol Laboratories, Inc., a subsidiary of the Baxter Laboratories, Inc., Morton Grove, Illinois, for providing sodium L-thyroxine pentahydrate and sodium D-thyroxine pentahydrate; to the Smith, Kline and French Laboratories, Philadelphia, for supplying triiodothyronine; and to Dr. T. C. Bruice, Dr. R. J. Winzler, and Dr. N. Kharasch for the deaminothyroxine used. The latter two compounds were originally provided by the Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

[2] Thyroxine crystals, Squibb.

buffered contents of the incubation vessels. Glucose and colloidal cholesterol,[3] prepared as previously described (1), were used as substrates.

Results

L-Thyroxine at concentrations ranging from 7.5×10^{-5} M to 2.5×10^{-5} M accelerated the rate of glucose oxidation by resting cells of *A. aerogenes* (Table I). The effect was observed only if the cells were preincubated with thyroxine for 30 minutes before tipping in the glucose. Under the conditions of these experiments, L-thyroxine did not significantly enhance

TABLE I

Effects of L-*Thyroxine on Oxidation of Glucose and of Cholesterol by A. aerogenes*

L-Thyroxine	Substrate	Substrate concentration	Mean Q_{O_2} and standard error, μl. per hr. per mg. dry weight	
			Without substrate	With substrate
M × 10^{-5}		M × 10^{-4}		
7.5–2.5	Glucose	2	4.4 ± 1.4	29 ± 1.0
0			2.3 ± 1.3	17 ± 1.0
7.5–2.5	"	3	3.5 ± 1.2	36 ± 1.9
0			2.3 ± 1.9	28 ± 1.3
20 –7.5	Cholesterol	2	1.5 ± 1.5	12.3 ± 0.9
0			1.0 ± 0.7	4.0 ± 2.0

Each flask contained 0.8 ml. of a bacterial suspension (1.1 mg. dry weight of cells) in 0.025 M phosphate buffer, pH 7.0, glucose or cholesterol, the sodium salt of L-thyroxine as indicated, and distilled water to a final volume of 2.0 ml. The center well contained 0.1 ml. of 20 per cent (weight per volume) KOH and a fluted filter paper. Seven experiments were performed at each concentration.

the autorespiration. D-Thyroxine, at the same concentrations, had no effect on the rate of glucose oxidation.

Similar experiments were carried out with colloidal cholesterol as the substrate. The oxidation of this sterol by the same bacterial system was relatively slow and erratic, in agreement with earlier observations by the authors (1) and by Stadtman *et al.*, who studied the oxidation of cholesterol by a soil *Mycobacterium* (3). However, L-thyroxine, at concentrations ranging from 2.0×10^{-4} M to 7.5×10^{-5} M, significantly enhanced the oxidation of the sterol under the conditions described above (Table I).

A few experiments were run with DL-thyroxine under similar conditions. Preliminary results indicate that cholesterol oxidation is stimulated by DL-thyroxine at concentrations of 4.5×10^{-4} to 3.5×10^{-4} M.

The effects of two thyroxine analogues, L-triiodothyronine and deamino-

[3] Cholesterol, Merck, U. S. P.

thyroxine, were investigated under similar conditions. L-Triiodothyronine accelerated the oxidation of glucose without preincubation and at concen-

TABLE II

Effects of L-*Triiodothyronine on Oxidation of Glucose by A. aerogenes*

L-Triiodothyronine	Q_{O_2}, μl. per hr. per mg. dry weight	
	Without glucose	With glucose
$M \times 10^{-5}$		
1.9	0	35
1.6	0	41
0	6	28
1.6	3	41
0.8	0	37
0	10	28
1.2	0	42
0.8	0	37
0	4	30

The incubation flasks were prepared as described for Table I, except that L-triiodothyronine was used instead of thyroxine, and the glucose concentration was 3×10^{-4} M.

TABLE III

Effects of Deaminothyroxine on Oxidation of Glucose by A. aerogenes

Deaminothyroxine	Q_{O_2}, μl. per hr. per mg. dry weight	
	Without glucose	With glucose
$M \times 10^{-6}$		
80	0	33
40	0	37
0	4	25
2.0	3	38
0	7	28
0.07	0	39
0	6.5	29

The incubation flasks were prepared as described for Table I, except that deaminothyroxine was used instead of thyroxine and the glucose concentration was 3×10^{-4} M.

trations slightly lower than those at which L-thyroxine was active (Table II). Cholesterol oxidation was also stimulated by triiodothyronine. The

effect was a temporary one, however, and fell off sharply after the first 15 or 20 minutes.

Deaminothyroxine also stimulated the oxidation of glucose by the *Aerobacter*. No preincubation was necessary. This compound was effective over a range of concentrations of 8×10^{-5} to 6.5×10^{-8} M (Table III). No further tests were made to determine whether any activity was evident at even lower concentrations.

DISCUSSION

The results reported indicate that L-thyroxine and a number of related compounds stimulate bacterial oxidations. L-Thyroxine is active in such systems over only a relatively narrow range of concentration. Although the minimal effective concentrations of the other compounds were not determined exactly, it is obvious that triiodothyronine accelerates glucose oxidation at somewhat lower levels and that deaminothyroxine is active at even lower concentrations. The relative potencies of these compounds in animal systems follow a similar pattern (4–6). It is possible that the differences in activity of the optical isomers of thyroxine and of the analogues are in part related to differences in their ability to penetrate the cell wall. This explanation seems plausible in view of the finding that triiodothyronine and deaminothyroxine are active without preincubation, while preincubation and higher concentrations are needed for the activity of thyroxine.

A number of observations in the literature support the hypothesis that thyroxine and other substituted phenols such as 2,4-dinitrophenol may act by means of uncoupling phosphorylation from the oxidative process (7–10). The bacterial system described above may provide a useful tool to study this problem further.

SUMMARY

The effects of L-thyroxine and a few related compounds on the oxidation of glucose and cholesterol by resting cells of *Aerobacter aerogenes* were studied by use of a Warburg apparatus.

The oxidation of glucose by this system was significantly stimulated by L-thyroxine, by triiodothyronine, and by deaminothyroxine, but not by D-thyroxine; triiodothyronine and deaminothyroxine were active at somewhat lower concentrations.

The bacterial oxidation of cholesterol was enhanced by L-thyroxine and DL-thyroxine under similar conditions.

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STUDIES ON CATALASE INHIBITION AS RELATED TO TUMORS*

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The inhibition of catalase *in vitro* by boiled extracts of tumors has been recently demonstrated (1). It was also found that fresh liver and spleen contained small amounts of a factor having similar action. In further work it was found that autolyzed liver homogenates contained large amounts of such inhibitory material. Studies on the rate of formation in liver homogenates, attempts at purification, and studies directed toward the elucidation of the possible nature of the responsible factor have been carried out.

EXPERIMENTAL

Homogenates of perfused beef and rat livers and the Jensen rat sarcoma served as the tissues for the preparation of boiled aqueous extracts (BAE) containing catalase inhibitor. Beef liver catalase (2) crystallized five times was utilized in studies with pure enzyme. The so called rapid (3), classical (4), and inverse (1, 5) methods of catalase assay were employed. In the last procedure the substrate is added at zero time after a suitable incubation period of the diluted enzyme with buffer. Catalase inhibition by BAE or other substances was determined by incubation of test material with a suitable amount of purified catalase for 2 hours at 2° prior to enzymatic assay. A control sample of catalase was always studied to correct for the activity losses of catalase in dilute solutions. The catalase activity of liver is expressed as the units of enzyme per gm. of dry weight of perfused tissue, as used previously (1).

Results

Effect of Aging Liver Homogenates on Catalase Activity—Fresh perfused liver was homogenized in a Waring blendor, and the gross tissue portions were removed by filtration through gauze. A sample was then assayed for catalase activity by the rapid and inverse methods over an extended period

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of time. The results for rat liver, Fig. 1, *A*, reveal a marked decrease in enzyme activity with time. As seen from the data of Fig. 1, *B*, a good deal of this apparent activity decrease is reversible with dilution. The characteristic rise in assay value with time during titrations by the rapid and classical methods has been observed previously in studies on BAE-catalase mixtures (1) and with lysates of sea-urchin embryo (5). The time required for the maximal reversal of enzyme activity as measured by the inverse method, *i.e.* the period during which the diluted homogenate is allowed to stand in buffer prior to addition of the hydrogen peroxide substrate, increases with the age of the homogenate. At the 24 hour period 4 minutes of incubation of the homogenate in buffer were sufficient to give maximal activity, 60 minutes being required at the 10th day. Previous studies of

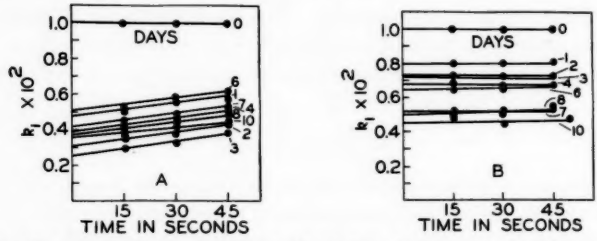

FIG. 1. Catalase activity of 0.07 ml. of rat liver homogenate at 22° measured by (*A*) the rapid method, and (*B*) the inverse method.

the effect of contact time of tumor BAE and purified catalase have demonstrated a similar relation (1).

The difference in the catalase activities of the homogenate found by the direct and inverse assays should be a function of the catalase inhibitor formed. A plot of this data is presented in Fig. 2, *A*. BAE homogenates were prepared at intervals and assayed for their ability to inhibit catalase. The results, presented in Fig. 2, *B*, reveal qualitative agreement with the inhibitor curve of Fig. 2, *A*.

The same effect that was noted for rat liver was also found to obtain for the bovine system. From Fig. 3 it can be seen that the catalase activity of this system was quite constant for the first 5 days and then suddenly decreased. The reversal effect was particularly striking at this time.

Purification Studies—The boiled aqueous extracts of Jensen rat sarcomas have higher levels of catalase inhibitor than those of aged liver homogenates and were consequently used in this work. The 80 per cent ethanol-soluble portion of these extracts, prepared as previously described (1), was utilized. The ethanol solution was passed through a Dowex 50 column without loss of inhibitor activity. The filtrate from a Dowex 2 column was inactive,

but attempts to elute catalase inhibitor were unsuccessful. Paper chromatography of the Dowex 50 filtrate was attempted. Difficulties of identification of active material in the eluates and of utilizing sufficient material on the paper to demonstrate clearly catalase inhibition made these experiments inconclusive. A disconcerting feature in attempts at purification of

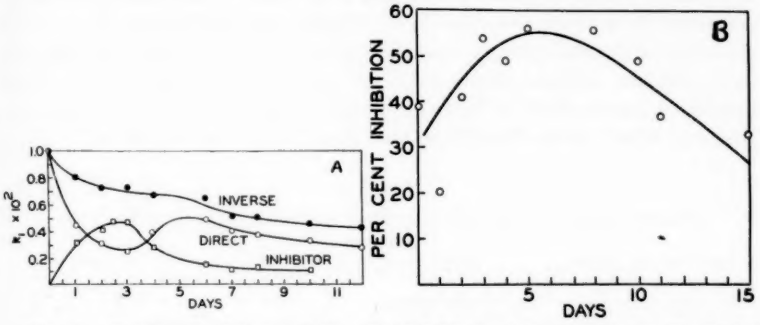

FIG. 2. Catalase inhibitor activity of (*A*) 0.07 ml. of homogenate, as illustrated by the difference in results of the direct (rapid) and inverse assay methods, and (*B*) 0.3 ml. of liver homogenate of BAE on an amount of crystalline beef liver catalase (1.5×10^{-9} M) sufficient to give a k_1 of 2×10^{-2} by the rapid method.

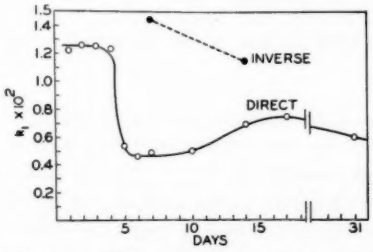

FIG. 3. Catalase activity of 0.03 ml. of beef liver homogenate at 22° measured by the direct (rapid) and the inverse methods.

the inhibitor is the rapid loss of activity. This appears to be oxidative in nature. Evaporation of inhibitor preparations with small amounts of H_2O_2 completely destroy the activity. This cannot be restored by treating with hydrogen in the presence of a palladized charcoal catalyst. Because of the extreme lability of the inhibitor following preliminary concentration to approximately 10 times the activity of starting BAE, this work was temporarily abandoned.

Effect of Sulfur-Containing Amino Acids on Liver Catalase Activity in Vivo—Sulfide and sulfhydryl compounds such as glutathione and cysteine

are known to be strong inhibitors of catalase (6–9). Several reports suggestive of a difference in sulfur-containing compounds of normal and tumor tissue are of interest in this respect. While the total sulfur content of tumors does not differ significantly from that of normal tissues, there is a marked increase in the alkali-labile sulfur (10). In liver tumors this is reflected in an increase in cystine and a decrease in the methionine level compared to the normal tissue (11). It was found that in rats given small amounts of cystine intraperitoneally there were substantial decreases in liver catalase activity compared with saline-treated controls. Typical results are summarized in Table I. Similar experiments with alanine, methionine, cysteic acid, glutathione, and cysteine showed no effects.

TABLE I

Effect of Intraperitoneal Administration of Cystine on Level of Rat Liver Catalase

No. of rats	Sex	Cystine injection		Catalase activity*		Per cent lowered
				Experimental	Controls	
		mg.	*days*			
5	♀	25	3	138	96	31
4	♂	25	5	281	175	38
5	♂	5	5	282	217	23

* Mean average in units per gm. of dry liver at 12 to 20 hours after the final cystine injection.

The depression of liver catalase activity by cystine is not as marked as that usually seen in the case of rats bearing large and rapidly growing tumors. This could possibly be due to various factors, including the cachexia associated with rapid tumor growth. Fasting has been reported to lower the liver catalase activity of rats (12) and mice (13). A similar effect is observed in rats on protein-free diets (14). In an experiment designed to determine whether fasting would augment the effect of cystine, a group of male rats weighing 72 to 80 gm. was placed on a restricted diet for 31 days prior to the daily intraperitoneal administration of 25 mg. of cystine for 5 days. The rats on the restricted diets gained an average of 40 gm. compared to 157 gm. for the controls. In the cystine-treated animals there was a 26 per cent depression of liver catalase activity compared with the restricted controls. This is the range found for rats fed *ad libitum* (see Table I) and indicates that growth restriction does not augment the effects of cystine. Dietary cystine supplementation sufficient to provide an additional 40 mg. daily over the controls caused no depression in liver catalase. Experiments of the latter type must be carefully controlled, for,

as shown in Fig. 4, the catalase activity of normal rats varies considerably with the age and weight of the animal.

The above experiments clearly demonstrated that cystine *in vivo* was a good inhibitor of liver catalase activity. Microbiological assays of the blood serum of normal rats and rats bearing large Jensen sarcomas for cystine and methionine, however, reveal no differences. The methionine level of both groups ranged from 0.8 to 1.0 and the cystine from 1.4 to 1.5 mg. per cent.[1]

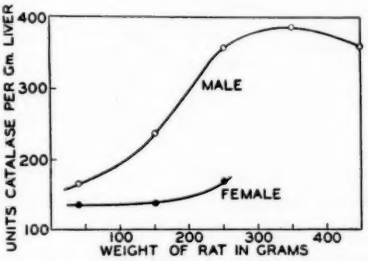

FIG. 4. Catalase activity of the livers of normal rats of different weights

TABLE II

Effect of Cystine on Catalase Activity of Homogenate of Perfused Rat Liver*

	Incubation time				
	0 hr.	1 hr.	2 hrs.	3 hrs.	8 hrs.
Homogenate only.....	1.0			0.93	0.98
" + cystine.....	0.95	0.90	0.84	0.75	0.73

* Activities expressed as first order reaction constants $\times 10^2$ given by 0.04 ml. of homogenate at 22° assayed by the rapid method.

Catalase Inhibition in Vitro—Cystine does not inhibit the activity of crystalline catalase. However, when it is added to liver homogenate, a definite inhibition results (Table II). A pronounced odor of H_2S can be detected within a few minutes after the cystine addition, owing to the well known desulferase activity of liver. The level of inhibition (25 per cent) is near that found *in vivo* in studies with cystine. Our result with cystine is in contrast to the work of Balls and Hale (15) who found that cystine activated the catalase of liver extracts.

In further preliminary experiments it was found that cysteine, glutathione, and sulfide markedly inhibited purified catalase, whereas cysteic

[1] The authors wish to thank B. E. Kline for these assays.

acid and alanine were without effect. Since both cysteine and sulfide are readily formed by liver tissue from cystine more detailed experiments utilizing the latter two compounds were carried out in an attempt to deter-

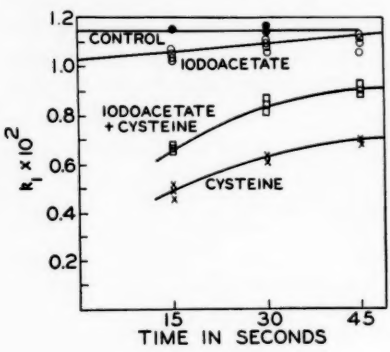

FIG. 5

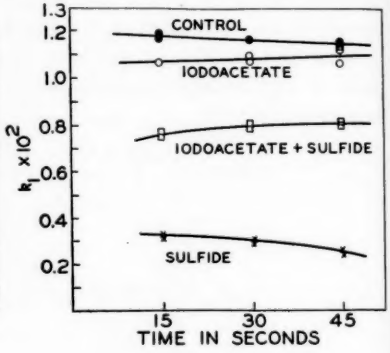

FIG. 6

FIG. 5. Effect of iodoacetate (0.053 M) on cysteine (0.026 M) inhibition of catalase (7.8×10^{-10} M).

FIG. 6. Effect of iodoacetate (0.1 M) on sulfide (0.02 M) inhibition of catalase (7.9×10^{-10} M).

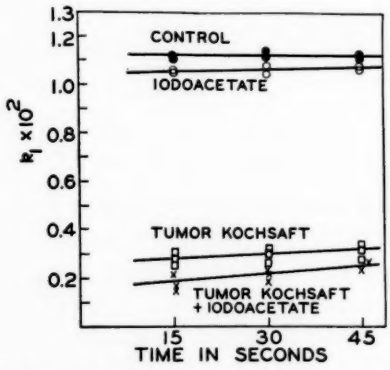

FIG. 7. Effect of iodoacetate (0.1 M) on inhibition of catalase (7.8×10^{-10} M) by tumor BAE.

mine whether these substances might be responsible for or related to the active material of BAE of tumors and liver autolysates.

Reversal of Cysteine and Sulfide Inhibition of Catalase by Iodoacetate—Both sulfide and cysteine strongly inhibit catalase. Iodoacetate has a slight inhibitory effect on catalase, but, as shown in Figs. 5 and 6, strongly

counteracts the inhibitory effects of cysteine and sulfide. In contrast, iodoacetate is ineffective in reversing the inhibition of catalase by BAE. Fig. 7 clearly illustrates that in this case the inhibitory effect of iodoacetate is additive to that of the BAE. Thus the action of the latter material does not appear to be due to sulfide or to a sulfhydryl compound. Further proof of this is seen in the failure of iodoacetate to elevate the catalase activity of homogenates prepared from the livers of rats bearing tumors.

DISCUSSION

The presence of a substance in BAE of aged liver homogenates capable of inhibiting catalase *in vitro* in a manner similar to that of BAE of tumors (1) and sea-urchin lysates (5) provides a ready source of material for study.

The depressing action of cystine on liver catalase activity *in vivo* is interesting in view of the apparent rise of this amino acid in tumor tissue (10, 11). The daily intraperitoneal administration of as little as 2 to 3 mg. per 100 gm. of rat over a 3 to 5 day period leads to definite depression of liver catalase activity. It appears that cystine is converted by the liver into an at present unknown active compound. Whether the substance in the BAE discussed here is the same as that formed by tumor tissue is not known. The work of other investigators on tumors has only provided material capable of inhibiting catalase *in vivo* (16–19).

The question as to whether a tumor depressed the concentration of catalase or merely its activity is still a matter of speculation. It would appear that a means of measuring catalase accurately independent of activity would be of great value in the study of this problem.

SUMMARY

1. Aging liver homogenates in the cold results in the formation of a heat-stable substance capable of inhibiting catalase.

2. The inhibitory material is relatively labile, which has to the present time made its concentration difficult.

3. The intraperitoneal administration of cystine to rats markedly lowers liver catalase activity.

4. Iodoacetate can partially reverse the inhibition of catalase activity by sulfide and cysteine *in vitro*, but is ineffective against boiled aqueous extracts of tumors.

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IMMUNOCHEMICAL STUDIES AND ASSAY OF CATALASE*

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Determinations of the level of an enzyme in biological material have been based for the most part on activity measurements. In quantitative work these have been referred to the activity of the purified enzyme. Since many factors may affect enzymatic catalysis in crude systems, a more direct method of estimation is needed. The quantitative immunochemical approach immediately suggests itself with respect to enzymes that are available in a high degree of purity. In a preliminary approach to this general problem crystalline catalase was utilized. Some of the reactions of this enzyme with rabbit antiserum have indicated the feasibility of this type of enzyme assay and thrown further light on the question of enzyme inhibition by antibody and the solubility of the antigen-antibody complex.

EXPERIMENTAL

Catalase crystallized five times was prepared from thoroughly perfused beef liver by a method involving preliminary fractionation of homogenates with cold acetone. The preparation gave a single boundary in the Spinco ultracentrifuge and a sedimentation constant at infinite dilution of 11.1×10^{-13} cm. per second. The partial specific volume was 0.725. If the diffusion constant of 4.1×10^{-7} sq. cm. per second for horse red cell catalase (1) is used, a molecular weight near 236,000 is calculated. Iron was measured by the sulfosalicylic acid method (2). A value of 0.102 per cent was obtained, from which a molecular weight of 220,000 is calculated if one assumes 4 atoms of iron per mole of protein. Assay of hematin by the alkaline pyridine hemochromogen procedure (3) revealed that each catalase molecule contained 3.2 hemes. This is in the range found by other investigators (4–6). The molecular extinction coefficients (β) at 278 and 405 mμ were 77.8×10^7 and 70.3×10^7 sq. cm. per mole respectively.

The measurements of *Kat. f.* were made by the method of von Euler and Josephson (7) and by the rapid method of Bonnichsen, Chance, and Theorell (8). In this study the former method will be referred to as the "classi-

* This study was supported in part by a grant from the Wisconsin Alumni Research Foundation.

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cal" procedure. The catalase preparation employed gave a *Kat. f.* of 51,000. Based on a catalase molecule of four hematin groups, a value of 64,000 is obtained. This is considerably higher than the activities usually reported for beef liver catalase (9–11).

Immunochemical Procedures—Rabbits were immunized by the adjuvant technique of Freund and McDermott (12). The γ-globulin fraction of the serum was separated by the method of Nichol and Deutsch (13) and reconstituted in an isotonic saline-borate buffer of pH 7.4 to the desired precipitin potency. The quantitative reactions were carried out under the conditions previously described (14).

Hemolysates—Beef erythrocytes were washed three to four times with 1 per cent NaCl and then suspended to their original volume with water containing approximately 0.5 per cent saponin. Following this, the stroma was removed by centrifugation. The supernatant solutions were thoroughly dialyzed against the saline-borate buffer, subjected to high speed centrifugation to remove suspended material, and then passed through a fine filter. Such hemolysates proved satisfactory for quantitative immunochemical studies. Those prepared by freezing and thawing techniques, by simple dilution hemolysis, or by use of lipoidal solvents such as ether, chloroform, and toluene were found unsuitable for immunochemical study, for they formed variable amounts of non-specific precipitate on standing in the cold.

Results

Quantitative Precipitin Reaction—The average results of four experiments for the reaction of the catalase, crystallized five times, with rabbit antibody are reported in Table I. A clear-cut equivalence zone was found, but the amount of antibody precipitated does not follow the Heidelberger and Kendall (15) relation of

$$Ab_{pptd.} = 2RAn - \frac{R^2An^2}{Ab_{max.}}$$

where $Ab_{pptd.}$ is the amount of antibody (Ab) N precipitated per amount of antigen (An) N added. R is the ratio of the Ab to An nitrogen at the equivalence point and, in the system studied, is equal to 1.81. The maximal amount of Ab precipitated from 1 ml. of Ab prepared ($Ab_{max.}$) amounted to 190 γ of nitrogen per ml. The data for the precipitin reaction were plotted to give Fig. 1, and this curve was used as the reference in calculating the amount of catalase N in the hemolysates assayed.

Assay of Bovine Red Blood Cell Lysates—Five hemolysates containing from 18.5 to 25.8 mg. of protein nitrogen per ml. were assayed by the reaction of from 0.5 to 3.0 ml. with 1 ml. of the Ab preparation. After analysis

of the specific precipitate for nitrogen, the amount of catalase was determined by reference to Fig. 1. The data presented in Table II reveal that the assay values in most cases rise with increasing amounts of hemolysate

TABLE I

Quantitative Precipitin Reaction of Catalase with Rabbit Anticatalase γ-Globulin

Antigen N added	Total N pptd.	Supernatant tests		Antibody N pptd.	Antibody N calculated*
		Antibody	Antigen		
γ	γ			γ	γ
7.5	52.5	−	+	45	26
15.0	98	−	+	83	50
22.6	138	−	+	115	73
30.1	165	−	+	135	93
45.1	204	−	+	159	128
60.2	238	−	+	178	156
75.2	262	−	−	187	175
105	295	−	−	190	190
120	300	+	−		
150	263	+	−		
237	224	+	−		
395	182	+	−		
790	130	+	−		

* From the Heidelberger and Kendall (15) equation.

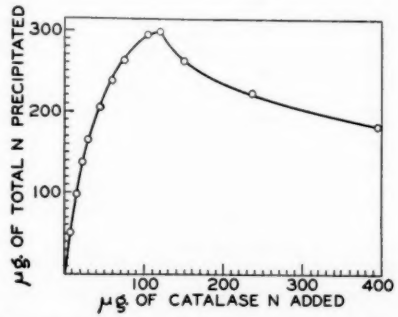

FIG. 1. Reaction of crystalline beef liver catalase with rabbit antibody

added. The result is opposed to what might be expected for an immunological cross-reaction. In such a reaction the amount of specific precipitation is always less than that given by the homologous antigen. If such a result was attained, the assay values would have shown a decrease with amount of hemolysate added. The per cent catalase was extrapolated to zero hemo-

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globin concentration. An example for this for Hemolysate A is presented in Fig. 2. The adequacy of this procedure was established by assaying a single level of Hemolysate A in the presence of increasing amounts of rabbit red blood cell lysate. The catalase of the latter system does not react with rabbit Ab to beef liver catalase. The data presented in Table III reveal a

TABLE II

Reaction of Bovine Red Blood Cell Hemolysates with Rabbit Anticatalase γ-Globulin

Sample assayed		Total N pptd.	Catalase N in ppt.	Catalase in hemolysate	Catalase concentration*
	ml.	*γ*	*γ*	*per cent*	*per cent*
A, 18.5 mg. N per ml.	0.5	49	6.5	0.070	0.068
	1.0	90	13.5	0.073	
	1.5	130	21.0	0.076	
	2.0	164	30.0	0.081	
	2.5	192	40.5	0.087	
	3.0	237	60.0	0.108	
B, 23.1 mg. N per ml.	0.5	69	10	0.087	
	1.0	141	21	0.088	0.073
	2.0	218	51	0.110	
	3.0	277	85	0.123	
C, 19.4 mg. N per ml.	0.5	53	7.5	0.077	
	1.0	97	14.5	0.075	0.077
	2.0	172	34	0.087	
	3.0	206	46	0.079	
D, 22.7 mg. N per ml.	0.5	76	11	0.097	
	1.0	134	22	0.097	0.093
	2.0	214	49	0.108	
	3.0	265	77	0.113	
E, 23.0 mg. N per ml.	0.5	68	10	0.087	
	1.0	133	21	0.091	0.084
	2.0	212	48	0.104	
	3.0	256	71	0.103	

* Determined by extrapolation of assay data to zero hemolysate concentration.

rise in the assay values with increasing amounts of added rabbit hemolysate. Apparently a high hemoglobin concentration has the effect of increasing the amount of precipitate formed. The rise is not due to occluded hemoglobin in the specific precipitate, for after three washings with isotonic NaCl these are visually free of hemoglobin.

In a preliminary experiment the application of this type of assay to the supernatant proteins of a homogenate of perfused calf liver yielded a catalase level near 1.5 per cent. This system likewise showed an apparent rise in catalase content with increasing amount of homogenate assayed.

Activity of Catalase in Hemolysate—The activity of these solutions was determined by the classical method. Variable assay results are often seen on aging hemolysates in the cold. Saponin in the concentrations employed did not appear to be responsible for this variation. After dialysis of the hemolysate there is usually a marked fall in the enzymatic activity. Calculation of the *Kat. f.* of Sample D, based on the amount of catalase determined immunologically, is as follows: 0.03 ml. of a 1:10 dilution of the

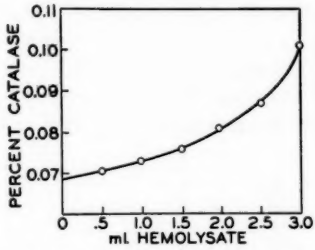

FIG. 2. Effect of hemolysate level on the immunochemical assay of catalase

TABLE III

Effect of Rabbit Hemolysate on Assay of Bovine Red Blood Cell Lysate

Bovine hemolysate*	Rabbit hemolysate†	Total N pptd.	Catalase N in ppt.	Catalase in hemolysate
ml.	*ml.*	γ	γ	*per cent*
1	0	90	13.5	0.072
1	1	98	15.0	0.081
1	2	123	19.5	0.105
0	1	0		
0	3	2		

* Contained 18.5 mg. of N per ml.
† Contained 25.8 mg. of N per ml.

hemolysate gave a k_1 of 3.3×10^{-2} when assayed by the classical method. After 36 hours dialysis in the cold, 0.04 ml. of a 1:10 dilution gave a k_1 of 2.4×10^{-2}. Sample D contained 0.0227 gm. of N per ml., of which 0.093 per cent was catalase. With a nitrogen conversion factor of 6.25, it is found that 0.03 and 0.04 ml. of a 1:10 dilution of Sample D contained 3.97 and 4.39×10^{-7} gm. of catalase, respectively. From the above data *Kat. f.* values of 83,000 and 45,000 for catalase of the hemolysate before and after dialysis are obtained. Sample E gave values of 80,000 and 55,400. The *Kat. f.* of Sample A determined after dialysis was 99,000. Samples B and C were not studied in this respect. Despite the technical difficulties ex-

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perienced in the enzymatic assays, the *Kat. f.* values in the original hemolysates of Samples D and E and for the dialyzed hemolysate of Sample A are found to be higher than the activity of the purified and crystalline enzymes. Difficulties in the enzymatic assay and activity losses with time result in low values; hence the *Kat. f.* of native catalase is very likely higher than that found here and probably in the range of high activity catalases previously reported (16–19). If the hemolysate contained protein possessing no enzymatic activity, but reacting immunologically as catalase, the *Kat. f.* values reported for this enzyme in the native system would also be low.

Solubility of Antigen-Antibody Complex—The system under consideration offers a unique method for this determination. It was carried out as follows: A specific precipitate containing 262 γ of N was formed at the equiv-

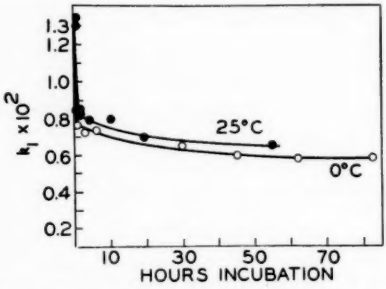

FIG. 3. Enzymatic activity of catalase in the presence of rabbit anticatalase

alence point. This was washed, uniformly suspended, and equilibrated with stirring for 20 hours at 37°. The suspension was then assayed for catalase activity by the classical method, 0.02 ml. giving a k_1 of 2.8×10^{-2}. The specific precipitate was removed by centrifugation and the supernatant solution assayed for enzymatic activity. 2 ml. gave a k_1 of 0.7×10^{-2} or only 0.25 per cent of that of the suspension on a volume basis. If we assume that the activity in the supernatant fluid is due to a soluble catalase-antibody complex and is the same as that of the specific precipitate, it can be readily calculated that the supernatant solution contains 0.22 γ of N per ml. If a part of the activity is due to catalase formed by dissociation of the antigen-antibody complex, the solubility of the specific precipitate would probably be even lower. When this experiment was repeated at 0°, the solubility was 0.20 γ of N per ml. In agreement with previous investigators, we found that temperature variations have little effect on the solubility of the specific precipitates of rabbit antibody. The extent of

solubility of the catalase is much lower than that reported for other antigen-antibody complexes (20–23).

Inhibition of Catalase by Antibody—Previous investigators (24, 25) have indicated that a suspension of the antibody complex of this enzyme retains almost complete activity. Assay of the suspended specific precipitate of catalase indicates that from 35 to 60 per cent of the original activity is lost. A non-specific γ-globulin had no effect on catalase activity when studied under analogous conditions.

The rate of inactivation of catalase by antibody at 25° and at 0° was followed by removing samples at intervals from an antibody-antigen mixture and assaying residual enzyme activity by the rapid method. These results, presented in Fig. 3, reveal that most of the loss in activity is almost instantaneous and varies little with temperature. The period of greatest decrease in activity is prior to the formation of a visible precipitate. Some variation in the percentage loss of enzymatic activity was encountered. This might be due to slight variations in the technique of addition of antibody to antigen.

DISCUSSION

In the preceding paper some of the factors which affect catalase activity in crude tissue extracts were discussed (26). Determination of the activity of this enzyme in red blood cell hemolysates results in considerable variation. The quantitative immunochemical assay appeared to be actually somewhat more satisfactory. Very small amounts of enzyme could be determined in systems containing in excess of 15 per cent hemoglobin. A real value of this type of assay is that it offers a method independent of activity and one that is strictly quantitative. It has also revealed that catalase in the native state has a higher activity than the usual crystalline material. This would explain why various investigators have been able on occasion to prepare catalases of activity in excess of the usually accepted *Kat. f.* levels of 50,000 to 60,000.

The immunochemical approach to the determination of the amount of enzymes in tissue extracts could find wide application in terms of those enzymes available in highly purified form. This supposes that a given enzyme will be identical in the different tissues of a given host. The ability to assay a beef hemolysate for catalase by means of an antibody to liver catalase indicates that the proteins from these sources are identical. A preliminary experiment by Bonnichsen (27) on the horse system is indicative of the same result.

This type of assay will be particularly adapted to deciding the question of whether the decrease in rat liver catalase occasioned by the presence of a

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rapidly growing tumor (28, 29) or the rise in activity due to testosterone administration (30) is merely a change in activity or a function of variation in actual enzyme concentration. Furthermore, it will offer a logical approach to deciding the question whether the higher animals actually show adaptive enzyme responses, as has been claimed (31, 32).

SUMMARY

1. Rabbit antibody to bovine catalase can be used to assay quantitatively red blood cell hemolysates for this enzyme.

2. The solubility of the catalase-antibody complex has been measured and found to be much lower than that reported for other antigen-antibody systems.

3. In contrast to previous work, it was found that a portion of the activity of catalase is inhibited by its antibody.

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AMINO ACID IMBALANCE AS RELATED TO TRYPTOPHAN REQUIREMENT OF THE RAT*

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In 1945, Krehl *et al.* (2) noted that the growth of rats was depressed by decreasing the casein content of a basal diet from 15 to 9 per cent through addition of 40 per cent of corn. The depression in growth could be corrected by addition of either niacin or tryptophan to the diet. Since this observation, numerous investigators have been concerned with factors that were considered to be related to the production of an increased requirement for niacin (3).

However, in a recent report from this laboratory by Salmon (3) on a restudy of the interrelationship between tryptophan and niacin, it was noted that under certain dietary conditions tryptophan, but not niacin, was effective in correcting growth depression in rats.

The present report is concerned with a detailed study of this initial observation in an attempt to determine basic changes that may occur in the tryptophan imbalance condition.

EXPERIMENTAL

Methods

Care of Animals—Weanling rats of either the Alabama Experiment Station strain or the Sprague-Dawley strain, 45 to 55 gm. in weight, were placed individually in wire-bottomed cages. Food and water were given *ad libitum*, and the animals were weighed weekly. Four or more rats were used per group, and essentially all experiments were repeated one or more times. The animals were fed the basal diets (Table I) or modifica-

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tions of them, as indicated, for a period of 4 weeks or longer. Records of food consumption were maintained on nearly all animals.

Determinations—Urine and fecal collections were made after the rats had been maintained on the respective diets and supplements for a period of at least 3 weeks. Samples were collected from individual rats placed in special metabolism cages over a period of 3 to 7 days. The urine samples

TABLE I

*Composition of Basic Diets**

The amounts represent gm. per kilo of diet, except for niacin which is in mg. per kilo of diet.

Ingredients	Diet I	Diet II	Diet III	Diet IV	Diet V
Extracted casein†	70	70	100	100	300
Oxidized casein‡				200	
Corn grits	400	400			
Gelatin		113			
Salts§	40	40	40	40	40
Sucrose	465	352	785	585	605
Corn oil	10	10	40	40	40
Cod liver oil	10	10	10	10	10
L-Cystine	3	3	3	3	3
Choline	2	2	2	2	2
Niacin			25	25	25

The following vitamins were added, in mg. per kilo of diet: α-tocopherol 25, α-tocopherol acetate 25, 2-methyl-1,4-naphthoquinone 5, inositol 1000, calcium pantothenate 30, thiamine 6, riboflavin 6, pyridoxine 6, folacin 1, biotin 0.5, and vitamin B_{12} 0.06.

* Subsequent changes in the diet as noted in the text were made at the expense of sucrose.

† Schaefer and Knowles (4).

‡ Hove, Copeland, and Salmon (5).

§ Salmon (6).

were made to a known volume with water, filtered, adjusted to pH 6.8, and refrigerated under toluene. Determinations for amino acids were made on both hydrolyzed ("total" amino acids) and unhydrolyzed ("free" amino acids) samples of urine. Aliquots of the urine samples were hydrolyzed with 2 N HCl or 2 N KOH in the autoclave for 8 hours at 15 pounds pressure, neutralized, made to volume, filtered, and refrigerated under toluene.

Heparinized blood samples were obtained from the animals at the termination of the experiments by heart puncture under light ether anesthesia. Food was removed from the cages 6 to 8 hours prior to obtaining the sam-

ples. Protein-free filtrates of the plasma were prepared immediately from the blood samples by essentially the procedure of Hier and Bergeim (7).

The amino acid analyses of the urine and plasma samples were performed by the microbiological assay methods previously employed (8–10). Fecal and dietary nitrogen was determined by the Kjeldahl (Gunning)

TABLE II

Growth of Weanling Rats Fed Casein-Grits Diets

Group No.	Basal diet No.	Treatment*	No. of rats	Average weight gain per rat per wk. (8 wk. period)	Average daily food consumption per rat	Average Hb values
		Experiment I. Alabama Experiment Station rats; average initial weight, 55 gm.				
				gm.	*gm.*	*gm. per 100 ml.*
1	I	7% casein	22	3.4	5.6	10.7
2	I	7% " + niacin	22	18.6	9.7	13.6
3	II	7% " + gelatin	10	0.9	4.4	8.4
4	II	7% " + " + niacin	14	12.4	6.9	13.6
5	II	7% " + " + " + tryptophan	6	21.4	10.1	15.0
6	II	7% casein + gelatin + tryptophan	3	22.8		
		Experiment II. Sprague-Dawley rats; average initial weight, 50 gm.				
7	I	7% casein	6	10.2		
8	I	7% " + niacin	6	24.9		
9	II	7% " + gelatin	6	1.4		
10	II	7% " + " + niacin	6	15.5		
11	II	7% " + " + " + tryptophan	6	35.3		

* When indicated, niacin was added at a level of 25 mg. per kilo of diet and DL-tryptophan at a level of 0.5 gm. per kilo of diet.

method. Corrections for endogenous nitrogen were made in the determination of protein digestibility by feeding animals nitrogen-free diets. Hemoglobin values were obtained by the oxyhemoglobin method.

Results

Effect of Tryptophan Imbalance on Growth—In the initial experiments, the corn grits-casein diets were employed. Results obtained with these diets are partly summarized in Table II. In Experiment I (Alabama Experiment Station strain of rats), it may be noted that the omission of the

niacin supplement from the diet caused a marked reduction in growth of the animals (Group 1 *versus* Group 2). The addition of gelatin to the niacin-deficient diet caused a further depression in growth (Group 3). Deaths occurred in groups maintained on this diet beyond the 8 week period. The depression in growth could be only partially restored by the supplementation of niacin to the diets (25 mg. per kilo) (Group 4). However, the addition of 0.05 per cent of DL-tryptophan to this diet completely

TABLE III

Effect of Tryptophan Imbalance on Growth of Weanling Rats

Group No.	Basal diet No.	Treatment*	Per cent of ingested nitrogen in feces	Average weight gain per rat per wk. (4 wk. period)†
		Experiment I. Alabama Experiment Station rats		
				gm.
1	III	10% casein		24.2
2	IV	10% " + 20% oxidized casein + M.		6.2
3	IV	10% " + 20% " " + T.		24.5
4	IV	10% " + 20% " " + " + M.		24.3
5	V	30% casein		26.5
		Experiment II. Sprague-Dawley rats		
6	III	10% casein	4.5	26.3
7	IV	10% " + 20% oxidized casein + M.	3.1	7.8
8	IV	10% " + 20% " " + " + T.	6.2	27.4
9	V	30% casein	4.2	35.6

* M. = DL-methionine, added at a level of 0.7 per cent of the diet; T. = DL-tryptophan, added at a level of 0.4 per cent of the diet.

† Average initial weight 47 gm.; four rats used per group.

restored normal growth. Such a condition that can be corrected by tryptophan, but not by niacin, has been termed in this report a "tryptophan imbalance." The above results appear to be in close agreement with the initial observation of Salmon (3).

In addition to the depression in growth, the omission of niacin from the diet produced a marked reduction in hemoglobin levels and severely fatty livers by the end of the 8 week period.

Similar results were obtained in Experiment II (Table II, Groups 7 to 11) in which the Sprague-Dawley strain of rats was used, indicating that the imbalance effect was not peculiar to the Alabama strain.

In later experiments, the casein-oxidized casein diets (Table I) were devised in an attempt to produce a more severe tryptophan imbalance and at the same time obtain a more purified diet with the elimination of corn grits. Results of growth experiments obtained with these diets are summarized in Tables III and IV. It may be noted that a marked depression in growth to the extent of 60 to 75 per cent occurred in the rats when 20

TABLE IV

Effect of Tryptophan and Niacin on Growth of Weanling Rats (Sprague-Dawley) Fed Tryptophan Imbalance Diet

Group No.	Basal diet No.	Treatment*	No. of rats	Average daily food consumption per rat	Average gain per rat per wk. (4 wk. period)†
		Experiment A. High niacin			
				gm.	*gm.*
1	III	10% casein	4	9.1	21.0
2	IV	10% " + 20% oxidized casein + M.	5	6.3	7.5
3	IV	10% " + 20% " " + " + T.	4	8.4	21.1
4	V	30% casein	4	10.3	30.9
		Experiment B			
5	III	10% casein	7	12.0	23.3
6	IV	10% " + 20% oxidized casein + M.	4	9.0	13.0
7	IV	Same as Group 6 + 0.025% L-tryptophan	4	11.0	17.5
8	IV	" " " 6 + 0.050% "	4	11.8	28.0
9	IV	" " " 6 + 0.160% "	4	12.0	30.0
10	V	30% casein	4	14.0	39.8

M. = DL-methionine, added at a level of 0.7 per cent of the diet in Groups 2, 3, 6 to 9; T. = DL-tryptophan, added at a level of 0.4 per cent of the diet in Group 3.

* In Experiment A, niacin was added at a level of 100 mg. per kilo of diet.

† Initial weight of rats, Experiment A, 45 gm.; Experiment B, 55 gm.

per cent of oxidized casein was added to the basal 10 per cent casein diet (Table III, Groups 1 and 2). For example, growth on the 10 per cent casein diet was about 25 gm. per week. Upon the addition of oxidized casein to the diet, growth of only 6 to 8 gm. per week was obtained. However, the supplementation of this diet with 0.4 per cent of DL-tryptophan corrected the imbalance condition and restored normal growth.

Since oxidized casein is devoid of cystine and methionine as well as tryptophan, the possibility exists that a methionine imbalance could occur in the diets tested. However, the omission of the DL-methionine supple-

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ment from the diet produced no retardation in growth (Table III, Group 3). The occurrence of a methionine imbalance may have been prevented by the methionine-sparing effect of the vitamin B_{12} and L-cystine added to the diet and by the level of casein used.

The tryptophan imbalance was produced readily with weanling rats of either the Alabama or the Sprague-Dawley strain (Table III). Older rats appeared to be less susceptible to the imbalance effects. It should be noted that the tryptophan imbalances were produced with diets contain-

TABLE V

Urinary Excretion of Amino Acids by Rats (Alabama Experiment Station) Fed Casein-Grits Diets

Diet	Average food consumption*	Per cent of ingested amino acids excreted in urine†			
		Tryptophan	Histidine	Phenylalanine	Methionine
	gm.				
7% casein	5.7	2.19 ±0.19 (21)	0.43 ±0.06 (10)	0.40 ±0.12 (5)	0.38 ±0.07 (6)
7% " + niacin	14.8	1.00 ±0.09 (21)	0.25 ±0.05 (10)	0.19 ±0.05 (5)	0.18 ±0.05 (5)
7% " + gelatin	5.4	2.44 ±0.39 (12)	0.39 ±0.08 (3)	0.65 ±0.15 (3)	0.35 ±0.09 (4)
7% " + " + niacin	9.4	1.67 ±0.15 (18)	0.18 ±0.03 (8)	0.11 ±0.02 (4)	0.09 ±0.01 (4)
7% casein + gelatin + niacin + tryptophan	16.2	‡	0.15 ±0.03 (4)	0.16 ±0.03 (4)	0.14 ±0.02 (4)

* Average daily food consumption per rat during urine collection period.

† Microbiologically "free" amino acids; average excretion ± standard error of the mean. The figures in parentheses indicate the number of individual samples analyzed.

‡ Omitted because of possible contamination of urine from the diet.

ing 25 mg. of niacin per kilo of diet. Even a level of 100 mg. of niacin per kilo of diet had no corrective effect on the growth depression produced by the tryptophan imbalance (Table IV, Groups 1 to 4). In contrast, graded levels of L-tryptophan afforded an increasing improvement in the growth of the rats on the imbalance diets (Table IV, Groups 5 to 10). A dietary supplement of 0.05 per cent of L-tryptophan to the imbalance diets permitted growth equal to the control animals fed the 10 per cent casein basal diet.

The amino acid imbalances did not appear to be the result of reduced digestibility and absorption of the protein. Little difference existed between groups on the various diets as to the percentage of ingested nitrogen appearing in the feces (Table III, Groups 6 to 9). The tryptophan im-

balance produced a marked lowering in the efficiency of gains of the animals, as may be noted from the ratios of the body weight gains to food consumption.

Effect of Tryptophan Imbalance on Urinary Excretion of Amino Acids—When niacin was omitted from the casein-corn grits diets, an increase in the per cent of ingested amino acids excreted "free" in the urine occurred. This increase was usually about 2-fold and statistically significant ($P = <0.01$). Urinary excretion of representative amino acids is presented

TABLE VI

Urinary Excretion of Amino Acids by Rats (Sprague-Dawley) Fed Tryptophan Imbalance Diets

Amino acid	No. of analyses	Per cent of ingested amino acids excreted in urine of rats fed*			
		10% casein	10% casein + 20% oxidized casein	10% casein + 20% oxidized casein + tryptophan	30% casein
Tryptophan, free†	14	0.75 ± 0.11	1.87 ± 0.18	‡	0.54 ± 0.06
" total	3	1.17 ± 0.31	3.56 ± 0.52		0.87 ± 0.08
Histidine, free	3	0.48 ± 0.13	0.48 ± 0.03	0.36 ± 0.06	0.29 ± 0.04
" total	3	1.28 ± 0.13	2.96 ± 1.78	1.22 ± 0.15	1.02 ± 0.26
Leucine, free	3	0.40 ± 0.14	0.54 ± 0.23	0.30 ± 0.03	0.17 ± 0.03
" total	3	0.55 ± 0.15	1.74 ± 0.81	0.78 ± 0.16	0.59 ± 0.17
Phenylalanine, free	3	0.30 ± 0.05	0.58 ± 0.15	0.26 ± 0.01	0.21 ± 0.03
" total	3	0.64 ± 0.06	1.61 ± 0.76	0.85 ± 0.09	0.76 ± 0.25
Food consumption§	14	13.6	6.7	12.4	16.4

* Average excretion ± standard error of the mean.

† Microbiologically "free" amino acids; total after hydrolysis.

‡ Omitted because of possible contamination of urine from the diet.

§ Average daily food consumption in gm. per day per rat during urine collection period.

in Table V. It may be noted that the degree of urinary loss of tryptophan, although limiting in the diet, was actually increased when niacin was omitted from the diet. Niacin or tryptophan supplement to the casein-corn grits diets reduced the urinary losses of amino acids.

The urinary excretion of amino acids by rats fed the oxidized casein imbalance diets was also increased 2- to 3-fold over that of control animals (Table VI). For example, the total urinary loss of histidine on the imbalance diet was 2.96 per cent of the ingested histidine compared to a loss of 1.22 per cent on the same diet supplemented with 0.4 per cent of DL-tryptophan. Control animals on the 10 per cent or 30 per cent casein diets excreted in the urine 1.28 and 1.02 per cent of the ingested histidine,

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respectively. Niacin was ineffective in preventing the urinary losses of amino acids. Again a significant increase in the urinary loss of tryptophan was noted in the rats fed the imbalance diet (0.75 to 1.87 per cent; $P = <0.01$).

TABLE VII

Amino Acid Content of Plasma of Rats Fed Tryptophan Imbalance Diets

Diet	Amino acid content of plasma, γ per ml.*				
	Tryptophan	Methionine	Valine	Glycine	Histidine
Experiment A. Alabama strain					
7% casein	26.0 ±2.7 (11)	13.7 ±0.8 (20)	32.3 ±2.4 (5)	23.9 ±1.4 (15)	11.6 ±0.7 (16)
7% " + niacin	39.5 ±2.9 (15)	13.4 ±0.5 (24)	27.0 ±1.8 (8)	18.7 ±1.2 (16)	11.2 ±0.6 (19)
7% " + gelatin	17.5 ±1.1 (3)	14.1 ±0.5 (5)	26.2 ±3.2 (2)	46.2 ±5.3 (5)	12.0 ±3.5 (5)
7% " + " + niacin	26.0 ±1.9 (11)	14.3 ±0.7 (19)	24.4 ±2.4 (4)	52.4 ±4.8 (17)	11.5 ±1.3 (11)
7% casein + gelatin + niacin + tryptophan	27.0 ±1.1 (2)	12.7 ±0.4 (14)	24.6 ±0.8 (4)	51.8 ±6.4 (13)	9.5 ±0.2 (11)
Experiment B. Sprague-Dawley					
10% casein	23.0 ±2.1 (12)	12.5 ±0.8 (12)	27.3 ±2.8 (12)	27.7 ±2.1 (7)	
10% " + 20% oxidized casein + methionine	11.6 ±1.0 (15)	11.1 ±0.6 (12)	36.0 ±4.2 (6)	26.2 ±4.6 (3)	
10% casein + 20% oxidized casein + methionine + tryptophan	34.3 ±1.7 (14)	16.7 ±2.0 (8)	35.0 ±3.7 (11)	18.8 ±2.7 (4)	
30% casein	32.7 ±2.9 (12)	17.3 ±1.5 (12)	31.7 ±3.6 (11)	21.0 ±2.4 (4)	

* Average ± standard error of the mean; the figures in parentheses indicate the number of individual samples analyzed.

Effect of Tryptophan Imbalance on Amino Acid Content of Plasma—The tryptophan imbalance produced a marked lowering in the free tryptophan level of the plasma. For example, in experiments employing the casein-corn grits diets, the omission of niacin significantly lowered the tryptophan content of the plasma from 39.5 to 26.0 γ per ml. (Table VII, Experiment A; $P = <0.01$). The addition of gelatin to the diet further reduced the tryptophan content of the plasma. The other amino acids determined

did not appear to be significantly affected by the omission of niacin from the diet. However, the addition of gelatin did increase considerably the glycine content of the plasma, reflecting perhaps the high glycine content of this protein.

In Experiment B (Table VII), the oxidized casein-tryptophan imbalance diet was used. The addition of the 20 per cent of oxidized casein to the basal 10 per cent casein diet produced a lowering of the free tryptophan content of the plasma (23.0 to 11.6 γ per ml.; $P = <0.01$). Supplementation of the imbalance diet with tryptophan increased the plasma level of tryptophan to that of control animals fed a 30 per cent casein diet (34.3 and 32.7 γ per ml., respectively).

The imbalance did not appear to affect greatly the other free plasma amino acids measured. The glycine level did appear to fall with an increased level of casein in the diet. Supplementary experiments confirmed this when it was noted that the free glycine in the blood progressively decreased when the casein level in the diet was progressively increased from 5 to 50 per cent.

DISCUSSION

From the results of the present study, it is apparent that the tryptophan requirement of the rat is not a constant factor, but is related to the diet employed and in particular to the protein or nitrogen level of the diet. When oxidized casein or gelatin, proteins devoid of tryptophan, was added to the basal diet, growth depression occurred that could be corrected by supplements of tryptophan. When 20 per cent of oxidized casein was added to the 10 per cent casein basal diet, an increase in the tryptophan level of the diet from 0.14 per cent to about 0.19 per cent of the diet was necessary in order to obtain comparable growth. This represents an increase of about 30 per cent in the tryptophan requirement of the rat when the protein in the diet was tripled. Salmon (3) found that the tryptophan requirement of the rat was 0.17 per cent of a casein-corn grits diet that contained 20 per cent protein and adequate niacin. When the diet contained only 10 per cent protein, the tryptophan requirement was only 0.10 per cent of the diet.

In the present study, niacin was without effect in correcting the imbalance. This can be explained readily on the basis that the basal diet contained adequate niacin, and hence no further sparing effect on tryptophan would be expected. Only under those conditions in which tryptophan is being used for niacin synthesis would the addition of niacin to the diet be expected to have an effect.

A number of possible explanations may be offered for the increased demand for tryptophan when the protein content of the diet is increased. The efficiency of utilization of the dietary tryptophan appears to be de-

pressed on the high protein diets, as was noted from ratios of food consumption and growth gains. The lowered utilization does not appear to be due to a lowered digestibility and absorption of the protein and tryptophan. It is conceivable, though, that a selective failure in tryptophan absorption could have occurred, although tryptophan analyses of the feces did not indicate such. It is recognized, of course, that intestinal bacterial action could have masked such possible effects.

However, the amino acid analyses of urine and plasma indicated that an imbalance condition existed. This was noted from the increased loss of tryptophan and other amino acids in the urine and from the marked lowering of the tryptophan level in the plasma. Other studies have revealed that amino acid deficiencies, vitamin deficiencies, and mineral deficiencies may greatly increase the urinary loss of amino acids by the rat or mouse (11–13). The increased loss of amino acids produced by the tryptophan imbalance may reflect an attempt by the animal to remove the excess unused amino acids. In so doing, tryptophan is also lost. However, the loss of tryptophan in the urine does not appear to be sufficiently great to account alone for the pronounced depression in growth of the animals.

The lowered plasma concentration of tryptophan is of interest in this respect. This effect, accompanied by the increased urinary loss of tryptophan, could very likely retard enzymatic ability and activity in protein synthesis. When tryptophan is added to the diet, a normal balance in the necessary plasma amino acids is obtained and protein synthesis can again proceed normally.

The amino acid imbalance and depression in plasma tryptophan may be related to an over-all effect of the tryptophan-low protein or to only specific amino acids in the protein. Such specific antagonisms have been noted in many instances with microorganisms (10, 14, 15). Similarly, the imbalance effect may be due to an increased or decreased protein turnover rate in the animals. Studies incorporating the use of radioisotopes are under way in this laboratory on these phases of the problem.

SUMMARY

1. An amino acid imbalance was produced by feeding a casein-gelatin diet or a casein-oxidized casein diet.

2. The reduction in growth of the animals due to the imbalance could be corrected by dietary supplements of tryptophan, but not by addition of niacin alone.

3. Protein digestion and absorption appeared to be similar for all groups.

4. The percentage of free and total tryptophan (and certain other amino acids) excreted in the urine by the rats on the imbalance diets was 2- to 3-fold over that excreted by control animals or by animals receiving supplements of tryptophan.

5. The imbalance produced a marked lowering of the plasma level of tryptophan, which was corrected by dietary supplements of tryptophan. The other plasma amino acids did not appear to be affected significantly.

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THE DARK AEROBIC METABOLISM OF ACETONE BY THE PHOTOSYNTHETIC BACTERIUM RHODOPSEUDOMONAS GELATINOSA*

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Among the *Athiorhodaceae* that are adapted to oxygen it has been observed by van Niel (1) that substrates which can be metabolized photosynthetically also can be metabolized aerobically in the dark. This observation has served largely as the basis for the conclusion that radiant energy is not involved in a direct activation of the substrate. An underlying assumption to this conclusion is that the same enzyme or group of enzymes is involved in the photosynthetic as well as the dark aerobic metabolism of the substrate.

The present investigation was undertaken to test the validity of this assumption for a particular substrate, acetone. Acetone was selected as the substrate for two reasons. First, the photosynthetic metabolism of acetone by *Rhodopseudomonas gelatinosa* was recently investigated and, second, alternative pathways for the metabolism of acetone have been established. Under photosynthetic conditions it was found that acetone condensed with CO_2 to form acetoacetate, which was split into 2 molecules of acetate (2). Under aerobic conditions, in the intact rat, it was found that acetone was oxidized to acetate and a 1-carbon fragment that behaved like formate (3). In addition, a soil diphtheroid has been isolated that oxidized acetone to acetol, an anticipated intermediate in the oxidative cleavage of acetone (4).

The results of the present investigation indicate that the same metabolic pathway is followed in the dark aerobic metabolism of acetone by *R. gelatinosa* as in its photosynthetic metabolism. Thus it may be inferred that the initial metabolic reactions are catalyzed by the same enzymes in either type of metabolism.

Methods

The bacteria were grown aerobically in the dark by procedures similar to those described by Kohlmiller and Gest (5). 1 liter Florence flasks were

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filled with 400 ml. of growth medium (6) containing 0.2 per cent acetone as the substrate, and inoculated with 10 ml. of a 2 to 3 day-old culture grown in a rich yeast extract medium. The flasks were sealed with sterile rubber stoppers, and each day they were opened aseptically to replenish the supply of oxygen. Rubber stoppers were used instead of cotton plugs to prevent loss of CO_2 and acetone. Each flask was wrapped with aluminum foil to exclude light, and the suspensions were aerated by shaking on a mechanical shaker. The incubations were carried out at about 30°. Under these conditions the growth rate was somewhat lower than under photosynthetic conditions.

After 48 to 72 hours of incubation, the bacteria were harvested by centrifugation, washed once with 0.05 M phosphate buffer, pH 7.0, and resuspended in 0.05 M phosphate buffer to a final cell density of 5 to 7 mg. of dry weight per ml.

Except for a few minor modifications, all other methods used were the same as those described previously (2). All incubations were carried out under aerobic conditions with air as the source of oxygen.

Results

Conversion of Acetone-1,3-C^{14} to Acetate—Resting cells, suspended in phosphate buffer, were incubated in the presence of acetone-1,3-C^{14} and acetate aerobically in the dark in modified Dixon-Keilin vessels. In an additional two vessels the acetate was omitted. After appropriate incubation periods the reactions were terminated by tipping in H_3PO_4 from a side arm, and the CO_2 was absorbed by opening the special stop-cock, containing KOH solution, to the center well. After dismantling the vessels, the KOH solutions were quantitatively transferred to small volumetric flasks and the cell suspensions to centrifuge tubes. The cellular material was removed and washed twice by centrifugation, and the supernatant solution and first wash were retained for the isolation of residual acetic acid. The acetic acid was isolated by steam distillation, the amount determined by titration with 0.01 N NaOH, and an aliquot assayed for C^{14}. In addition, the remaining fractions (CO_2, cellular material, and nonvolatile soluble substances) were assayed for C^{14} and represented unidentified metabolic products of acetone.

The results of this experiment are presented in Table I. When acetate was present as a trapping agent, 25 to 30 per cent of the total C^{14} metabolized was found in the volatile acid fraction. In the absence of added acetate, the same total activity was found in the metabolic products for an equivalent incubation period, but only a very small percentage of the activity was located in the volatile acid fraction. These results indicate that acetone was converted to acetate. The relatively high specific ac-

tivity of the residual acetate suggests that the acetate was an early intermediate in the aerobic metabolism of acetone.

Incorporation of $C^{14}O_2$ into Acetate—The extent of incorporation of $C^{14}O_2$ into acetate during the dark aerobic metabolism of acetone was investigated by incubating a resting cell suspension in phosphate buffer with acetone, acetate, and $NaHC^{14}O_3$. The non-specific incorporation of $C^{14}O_2$ into acetate was determined by controls containing acetate and $NaHC^{14}O_3$, but no acetone. The suspensions were treated in the same manner as the previous experiment.

The results of three separate experiments are summarized in Table II. In each case it will be noted that, in the presence of acetone, 10 to 15 times as much CO_2 was fixed (see the last column) as in its absence. Of

TABLE I

*Conversion of Acetone-1,3-C^{14} to Acetate**

Additions	Incubation period	Residual acetate	Specific activity of acetate	Total activity of acetate	Total C^{14} in metabolic products
	hrs.	*μmoles*	*c.p.m. per μmole*	*c.p.m.*	*c.p.m.*
Acetate	1.25	54.2	690	32,100	102,000
"	2.0	35.3	1110	39,300	121,000
None	0.75	Trace		330	63,500
"	1.25	"		510	99,400

* Each vessel contained initially 30 μmoles of acetone-1,3-C^{14} (specific activity, 7310 c.p.m. per μmole) and 12 mg. of cells (dry weight) suspended in 2.4 ml. of 0.04 M phosphate buffer, pH 7.0. Where indicated, 60 μmoles of acetate were added.

the total C^{14} fixed in the presence of acetone, as much as 70 per cent was associated with the volatile acid fraction. In the absence of acetone a much smaller fraction of the total C^{14} fixed was associated with the acetic acid. Thus when the specific activities of the acetic acid fractions are compared, it is noted that in the presence of acetone the values ranged from 35 to over 100 times greater than in its absence. Such differences could not be accounted for on the basis of excessive dilution of the $C^{14}O_2$ because, in each case, the final specific activity of the CO_2 with acetate as the only substrate was equal to or greater than the specific activity of the CO_2 in companion vessels containing acetone plus acetate.

Incorporation of $C^{14}O_2$ into Acetoacetate—Resting cell suspensions were incubated with acetoacetate and $NaHC^{14}O_3$ in the presence and absence of acetone. The suspensions were shaken in the dark in ordinary Warburg vessels fitted with two side arms. Incubation was terminated by acidification to pH 4 with H_3PO_4 and the liberated CO_2 was absorbed in KOH

added through the vent into one of the side arms. The KOH solution then was removed quantitatively from the side arm and the vessel was detached from the manometer. Remaining traces of $C^{14}O_2$ were removed by adding 0.2 ml. of 0.3 M $NaHCO_3$ solution to the suspension, followed by 2 minutes of aeration. The Warburg vessels were then reassembled with citric acid and aniline citrate, respectively, in the two side arms, and the residual acetoacetic acid was decarboxylated with aniline citrate. The

TABLE II

Formation of Labeled Acetate during Dark Aerobic Metabolism of Acetone in Presence of $C^{14}O_2$ and Acetate

Experiment No.*	Additions	Incubation period	Final specific activity of CO_2	Specific activity of acetate	Total activity of acetate	Total C^{14} fixed
		hrs.	*c.p.m. per μmole*	*c.p.m. per μmole*	*c.p.m.*	*c.p.m.*
I	Acetone	1.5	2370	333	13,440	25,600
	None	1.5	2680	5	70	1,700
	Acetone	2.5	1500	352	7,550	23,000
	None	2.5	1600	10	80	2,300
II	Acetone	1.0	6010	647	41,700	57,200
	None	1.0	5260	5	200	3,700
	Acetone	1.75	3110	832	57,700	90,300
	None	1.75	4260	21	700	6,500
III	Acetone	1.25	3100	283	10,700	40,500
	None	1.25	4000	6	105	4,310

* Experiment I, each vessel contained 8 mg. of bacteria (dry weight), 20 μmoles of $C^{14}O_2$ (specific activity, 5000 c.p.m. per μmole), and 40 μmoles of acetate in 0.04 M phosphate buffer, pH 7.0; total volume, 2.5 ml. Where indicated, 20 μmoles of acetone were added. Experiment II, each vessel contained 13 mg. of bacteria (dry weight), 20 μmoles of $C^{14}O_2$ (specific activity, 8640 c.p.m. per μmole), and 60 μmoles of acetate in 0.04 M phosphate buffer, pH 7.0; total volume, 2.5 ml. Where indicated, 40 μmoles of acetone were added. Experiment III, same as Experiment II, except that the specific activity of the $C^{14}O_2$ was 10,270 c.p.m. per μmole.

volume of liberated CO_2 was determined and later absorbed in KOH by the technique described above. The remainder of the fractionation procedure was the same as before with the addition that the acetone resulting from the decarboxylation of acetoacetate, plus any residual acetone, was recovered quantitatively. The acetone was subsequently degraded to iodoform and acetate.

The foregoing separation and degradations provided the following fractions: (1) residual $C^{14}O_2$, (2) CO_2 originating from the carbonyl group of acetoacetate, (3) iodoform originating from the methyl and methylene carbons of acetoacetate, (4) acetate originating from the carboxyl group

and the average of methyl and methylene carbons of acetoacetate, and (5) acetate produced metabolically during the incubation period. All fractions were assayed for C^{14} and the results converted to specific activities. In calculating the specific activity of the products derived from the acetone moiety of acetoacetate, only the amount which could have originated from the residual acetoacetate was considered.

The results of this experiment are presented in Table III. First, it will be noted that the specific activity of the acetoacetate carboxyl carbon was 11 to 17 times greater in the presence of acetone than in its absence. Second, it is observed that most of the activity was found in the carboxyl group of acetoacetate. Residual acetoacetate from the 0.5 hour incuba-

TABLE III

*Formation of Labeled Acetoacetate during Dark Aerobic Metabolism of Acetone in Presence of $C^{14}O_2$ and Acetoacetate**

Additions	Incubation period	Final activity of CO_2	Aceto-acetate carboxyl	Aceto-acetate carbonyl	Aceto-acetate methyl or methylene	Residual acetate
	hrs.	*c.p.m. per μmole*	*c.p.m. per μmole*	*c.p.m. per μmole*	*c.p.m. per μmole*	*c.p.m. per μmole*
Acetone	0.5	1920	322	5	1	120
"	1.25	1200	325	35	0.5	174
"	2.0	800				233
None	0.5	2050	28	1	1	6
"	1.25	1050	19	0	0.5	5

* Each vessel contained initially 50 μmoles of acetoacetate, 20 μmoles of $NaHC^{14}O_3$ (specific activity, 3850 c.p.m. per μmole), 12 mg. (dry weight) of bacteria, and 0.04 M phosphate buffer, pH 7.0, in a total volume of 2.5 ml. About 20 μmoles of acetone were added as indicated.

tion with acetone contained 65 times as much activity in the carboxyl position as in the carbonyl position. This ratio dropped to a value of 9 in the longer incubation period. Finally, it should be noted that the specific activity of the acetate that accumulated during the incubation period was roughly one-half of the specific activity of the acetoacetate carboxyl carbon. Degradation of the acetate revealed that all of the C^{14} was located in the carboxyl group.

DISCUSSION

The experiments with acetone-1,3-C^{14} indicate that the carbon atoms of acetone participate directly in the formation of acetate. Similarly the experiments with $NaHC^{14}O_3$ indicate that the acetate also is derived, in part, from CO_2. The formation of labeled acetate from $C^{14}O_2$ was found to occur only in the presence of acetone, and subsequently it was shown that

all of the activity was located in the carboxyl group. From these results it may be inferred that CO_2 is incorporated into acetate by the carboxylation of acetone or a derivative of acetone.

Two mechanisms can be postulated for the formation of labeled acetoacetate during the incubation of acetone and $C^{14}O_2$. One possibility is that acetoacetate is produced directly by the carboxylation of acetone (Equation 1) and then breaks down to form acetate (Equation 2).

(1) $$CH_3COCH_3 + C^*O_2 \rightarrow CH_3COCH_2C^*OOH$$

(2) $$CH_3COCH_2C^*OOH \rightarrow 2CH_3C^*OOH$$

The other possibility is that acetoacetate exchanges with labeled acetate (Equation 3), which is produced by the condensation of $C^{14}O_2$ with a 1-carbon fragment derived from acetone (Equations 4 and 5).

(3) $$CH_3COCH_2COOH + 2CH_3C^*OOH \rightarrow CH_3C^*OCH_2C^*OOH + 2CH_3COOH$$

(4) $$CH_3COCH_3 \rightarrow CH_3COOH + C_1$$

(5) $$C_1 + C^*O_2 \rightarrow CH_3C^*OOH$$

Superficially it would appear that the two mechanisms could be easily distinguished by determining the ratio of the C^{14} content of the carboxyl and carbonyl groups of the residual acetoacetate. According to Equation 1, all of the activity should be located in the carboxyl group, and according to Equation 3 the activity should be equally distributed between the two groups.

Two mitigating circumstances tend to obscure this difference. First, it has been observed that the splitting of acetoacetate to acetate by *R. gelatinosa* is a reversible reaction. In the presence of labeled acetate, produced by either mechanism, this results in the labeling of the carbonyl position of acetoacetate. Second, it has been shown that the incorporation of C^{14} into acetoacetate according to Equation 3 results in asymmetrically labeled acetoacetate. Recently Beinert and Stansly (7) found that, when acetoacetate and carboxyl-labeled acetyl coenzyme A were incubated with a partially purified enzyme system from pig heart, the activity of the carboxyl carbon of acetoacetate was 2 to 3 times greater than the carbonyl carbon. Similar values have been obtained with intact *R. gelatinosa* when carboxyl-labeled acetate was used instead of acetyl coenzyme A.[1] Thus either mechanism can result in asymmetrically labeled acetoacetate, although quantitative differences may be expected.

In the present investigation (Table III) the activity of the acetoacetate carboxyl carbon was 65 times greater than the activity of the carbonyl carbon. This ratio is much greater than the highest value observed for

[1] Unpublished data.

an exchange process. When the incubation period was increased to 1.25 hours, the ratio decreased to a value of 9. This may be ascribed to the greater participation of the exchange reaction during the longer incubation period. In the absence of added acetone, the residual acetoacetate contained less than one-tenth of the activity when acetone was present, but again it was observed that the distribution of C^{14} was highly asymmetric. The degree of asymmetry was considerably greater than that observed for a pure exchange process and was probably due to the presence of small amounts of acetone introduced as an impurity in the acetoacetate solution or produced by decarboxylation of acetoacetate during the incubation period.

The two mechanisms for the formation of labeled acetoacetate can also be distinguished by the relative specific activities of the residual acetoacetate and the accumulated acetate. According to Equation 2 the specific activity of the acetate, on a molar basis, should be one-half of the specific activity of the acetoacetate. On the other hand, if acetate was the precursor of labeled acetoacetate by an exchange reaction (Equation 3), the specific activity of the acetate should be greater than the specific activity of the acetoacetate, at least during the early part of the incubation period when the specific activity of the acetate is increasing. In the present investigation (Table III), it was found that the accumulated acetate had a specific activity slightly less than one-half of the specific activity of the residual acetoacetate. This result is consistent with the concept that the acetoacetate is the precursor of acetate and that the labeled acetoacetate was produced by the direct carboxylation of acetone.

As a result of these studies it may be concluded that the dark aerobic metabolism of acetone proceeds by the same pathway as in the photosynthetic metabolism. From this it may be inferred that the same enzymes are utilized in the initial metabolic reactions of acetone in either case.

The carboxylation of acetone to form acetoacetate does not represent an oxidation in the usual sense of the term, and therefore O_2 or light must be required to supply the necessary energy only. Since, under the two conditions, the same enzyme or enzymes are involved in the carboxylation reaction, the energy from either source must be converted to a common form prior to the actual involvement of the substrate. Thus it is highly unlikely that either O_2 or radiant energy is involved in a direct activation of acetone.

SUMMARY

The pathway of the dark aerobic metabolism of acetone by *Rhodopseudomonas gelatinosa* was investigated with C^{14}-labeling techniques. The results indicate that acetone undergoes direct carboxylation with CO_2 to

form acetoacetate, which is the precursor of acetate. This pathway coincides with that previously observed for the photosynthetic metabolism of acetone.

These findings are discussed in relation to the mechanism of bacterial photosynthesis.

Addendum—Since this paper was submitted for publication, Frenkel (8) has demonstrated a light-induced formation of adenosine triphosphate from adenosine diphosphate and orthophosphate in cell-free enzyme preparations of *Rhodospirillum rubrum*. This finding supports the conclusions reached in this paper.

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DIETARY EFFECTS ON LIPOGENESIS IN ADIPOSE TISSUE*

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In an earlier communication we compared lipogenesis from glucose in the hepatic and adipose tissue of rats fed a stock diet (1). The present study deals with the influence of the nutritional state of the animal on lipogenesis within adipose tissue. The results presented here confirm the superiority of lipogenesis in adipose tissue over that found in hepatic tissue and suggest that the liver may play a supplementary rôle in the synthesis of fat from carbohydrates. They indicate also that the rate of fat synthesis from carbohydrates is more markedly influenced by the fat than by the carbohydrate content of the antecedent diet.

EXPERIMENTAL

Treatment of Rats—Male Wistar rats weighing 300 gm. and more were used. Their weights were recorded for several weeks prior to the experiment, during which time they received stock diet. Only animals showing an average daily weight gain of 2 to 3 gm. were employed. The special diets were fed *ad libitum* for at least 14 days before sacrifice. The composition of the diets, including the stock diet (Purina dog chow checkers), and other data pertaining to the experiments are presented in Table I. The weight gain of most animals was slightly impaired during the periods in which the special diets were fed compared with the preceding period on the stock diet.

Incubation and Analytical Procedures—Preparation of the tissues and incubation techniques have been described elsewhere (1). Pooled tissues from three rats, *i.e.* 200 mg. from each, were used per flask. The medium contained uniformly labeled glucose in a final concentration of 400 mg. per cent. The incubation was conducted at 37° for 3 hours. A slight modification of the method of Masoro *et al.* (2) was used for the determination of the tissue fatty acids. The radioactivity of the evolved carbon dioxide and of the fatty acids was determined according to Entenman *et al.* (3).

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Results

Dietary Effects on Adipose Tissue Lipogenesis—The results, expressed in percentage conversion per gm. wet weight of tissue, are summarized in Table II. Lipogenesis was abolished during prolonged fasting and during

TABLE I

Composition of Diets and History of Rats

Experiment No.	Diet	Days fed	Composition of diets, gm. per 100 gm. diet			Average weight at sacrifice	Weight change per day
			Fat	Carbohydrate	Protein		
						gm.	gm.
1a, 1b	Fasted	8				322	−8.9
2a, 2b	A	14	60	1.2	26	369	−0.8
3a, 3b	B	14	35	34	15	341	+1.9
4a, 4b	C	15	13	55	15	338	+3.4
5a	D	22	0.8	70	15	395	+1.7
5b		22				418	+1.6
5c		14				327	−0.8
6a	Stock	*	6.3	4.7	27	405	+9.6
7a, 7b	A	16*	60	1.2	26	322	+10.7
8a, 8b	B	16*	35	34	15	327	+8.9

All diets but the stock diet contained 4 per cent Mendel-Osborne salt mixture, 4 per cent yeast, 0.3 per cent wheat germ, and cod liver oil each, and approximately 10 per cent fiber. Fat was given as Mazola, carbohydrates as sucrose, and protein as casein. The components contained in the oils and in the yeast are included in the figures of the table. The stock diet was Purina dog chow checkers.

* All rats received protamine zinc insulin (Lilly); on the 1st day 2 × 4 units, on the 2nd day 2 × 6 units, and on the 3rd day 8 units, about 3 hours before sacrifice.

feeding of Diet A, high fat. This diet resembles the quantitative composition of the metabolic fuels which are used by the organism during fasting. Diet B, containing equal amounts of fat and carbohydrate, induced a slight fat synthesis. The utilization of glucose for fat formation rose to a significant level when Diet C was fed, but it was below the value previously observed in rats fed the stock diet (1). Feeding the practically fat-free, high carbohydrate diet (Diet D) increased adipose tissue lipogenesis to the highest rate observed in normal rats.

There was little correlation between utilization of glucose for fatty acid synthesis and the rate of conversion to carbon dioxide.

Hepatic Glucose Utilization by Rats Fed Carbohydrate-Rich, Non-fat Diet—In three rats hepatic lipogenesis was determined after feeding Diet D.

TABLE II

Dietary Effects on Lipogenesis and Oxidation by Adipose and Hepatic Tissues

Experiment No.	Diet	Per cent tissue nitrogen	Per cent tissue fatty acids	Per cent glucose per gm. tissue recovered as	
				Fatty acids	CO_2
Adipose tissue					
1a	Fasting	0.56	47	0	0.56
1b		0.74	50	0	0.39
2a	A	0.17	79	0	0.52
2b		0.16	80	0	0.32
3a	B	0.17	79.7	0.14	0.33
3b		0.17	73.6	0.15	0.36
4a	C	0.18	75.0	0.96	2.9
4b		0.19	75.0	0.31	0.71
5a	D	0.23	80	4.1	2.6
5b		0.22	80	14.8	3.1
5c		0.23	79	1.85	2.1
6a	Stock*	0.21	75	3.9	3.1
7a	A*	0.16	82	0.8	0.43
7b		0.16	87	0.13	0.52
8a	B*	0.22	78	0.52	0.85
8b		0.17	77	0.35	0.70
Liver					
5a	D	3.0	7.8	1.25	3.7

* Insulin-treated; see foot-note to Table I.

It was lower than lipogenesis by adipose tissue from the same animals (Table II).

Effect of Insulin on Adipose Tissue Lipogenesis during Feeding of High Fat Diets—Administration of protamine zinc insulin for 60 hours to rats fed the stock diet produced an increased formation of fatty acids and CO_2. This effect was much less pronounced after feeding the high fat diets (Diets A and B, Experiments 7 and 8).

Influence of Mincing Adipose Tissue on Lipogenesis—The adipose tissue used in these experiments, that is, the thin peripheral part of the testicular

fat body, presents a rather intact anatomical structure, whereas the liver slices have two large injured surfaces. An experiment was performed to establish whether injury of the fat tissue by cutting produces significant change in its lipogenic activity. Two 600 mg. samples of testicular tissue were taken from one animal, and one was finely cut with scissors. The lipogenic activity of this minced tissue was compared with that of the thin part of the testicular fat body which was prepared in the usual manner. In addition, minced adipose tissue from other areas of the animal was investigated. The results are presented in Table III. The higher lipogenic activity of the brown, scapular fat tissue is probably related to the higher protein content of that tissue.

TABLE III

Lipogenesis by Adipose Tissue from Different Regions of Same Animal

	Non-minced, testicular*	Minced			
		Testicular*	Perirenal	Subcutaneous†	Scapular
% conversion to fatty acids per gm. tissue	1.47	1.40	1.07	0.95	2.28
% conversion to CO_2 per gm. tissue	1.25		0.9	1.4	6.6

* Thin peripheral part of testicular fat body.
† Inguinal.

DISCUSSION

Fasting or feeding a high fat diet abolished lipogenesis in adipose tissue and reduced glucose oxidation markedly. This observation is in agreement with the findings of Masoro *et al.* (4) on hepatic glucose utilization and the finding by Hausberger and Jachtorowycz (5) that adipose tissue of fasted rats has lost its ability to synthesize glycogen from glucose *in vitro*. Lipogenesis increased to the highest levels on a high carbohydrate, fat-free diet. Such a diet promotes not only especially high glycogen (6, 7) and total carbohydrate levels, but also increases glycolysis in adipose tissue (6). It is interesting to note that lipogenesis was greatest when the dilution by the endogenous carbohydrates present in the fat cells was presumably the highest. As a result of this metabolic dilution, our percentage conversion rates are probably minimal.

It should be noted that the caloric percentage of carbohydrates in Diet C was slightly greater than in the stock diet, which contained approximately half the amount of fat in Diet C. In earlier experiments (1) adipose tissue lipogenesis was found to be almost twice as high when the stock diet was fed as with the previous feeding of Diet C (Table II). The

rate of lipogenesis from available carbohydrates seems to be regulated by the fat of the food, as well as by the carbohydrate content *per se*. Our data suggest, as do those of Masoro *et al.* (4), that the protein content of the diet does not exert a major influence on lipogenesis.

Our finding that exogenous insulin during fasting or feeding a high fat diet has almost lost its ability to stimulate lipogenesis and oxidation of glucose indicates that the diminished glucose utilization during fasting or feeding a high fat diet is not caused by lack of insulin. Since the carbohydrate utilization of the hypophysectomized animal depends also on the animal's previous nutritional state (8), it may be assumed that the changes producing the altered responsiveness of the tissues are autonomous.

In an earlier study (1) we observed in rats fed a stock diet a very low hepatic lipogenesis, whereas the values of Masoro *et al.* (4) were about 50 times higher. However, the rats in their experiments received a fat-free carbohydrate-rich diet. We investigated fat synthesis by liver slices in three rats fed a similar diet (Diet D, Experiment 5a). Hepatic lipogenesis was 1.25 per cent, a value similar to that found by Masoro. Feeding the same diet also stimulated lipogenesis in adipose tissue, which again considerably exceeded that found in the liver. If the *in vitro* capacities of these tissues are a reasonable measure of their physiological rôles, one can hardly avoid the conclusion that fat synthesis by the liver is supplementary to that by adipose tissue. The liver may contribute to lipogenesis possibly only under special conditions. The assumption that adipose tissue is the main site of lipogenesis is supported by the observation of an unimpaired lipogenesis in functionally liverless, eviscerated rats (4). Hausberger (9) has expressed the opinion that adipose tissue may play an active and important part in fat and carbohydrate metabolism, an opinion amplified by the present studies. It seems quite possible that not only disturbances like certain forms of obesity are caused by a faulty mechanism of that tissue in which fat synthesis and deposition take place, but also the "lipoplethoric diabetes" described by Lawrence (10).

SUMMARY

Utilization of radioglucose by adipose tissue *in vitro* has been investigated under various nutritional conditions. Fasting or feeding of a high fat diet abolishes lipogenesis and greatly diminishes glucose oxidation. Pretreatment with insulin has little stimulating effect on glucose utilization in adipose tissue of rats fed a high fat diet. The rate of lipogenesis from available carbohydrates seems to be regulated not only by the carbohydrate content of the diet; glucose utilization *increases* as the carbohydrate content *increases* or the fat content *decreases*. The lipogenic activity of the adipose tissue was found to be superior to that of the liver of the same animals.

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THE PARTITION OF MANGANESE AMONG ORGANS AND INTRACELLULAR ORGANELLES OF THE RAT*

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The essential metabolic rôle of manganese in the animal organism has long been a matter of record. It was an early finding that intravenously injected manganous salts are removed quickly from the blood stream (1), leaving the body somewhat less rapidly by way of the biliary tract (2). In the course of studies on the kinetics of manganese, our observations led us to surmise the existence in the body of a mechanism whereby the manganese is temporarily diverted in transit, entrapped, and then gradually released. Such a mechanism, with the resulting rapid movement of the element into the entrapping system, would provide an explanation for the lack of agreement noted in previous studies of this metal's distribution in organs (3–7).

The isotope used in this study, Mn^{56}, has the advantage of being readily available to us in sufficiently high specific activity so that dosages need not exceed levels of normal tolerance. The short half life of this material confined our attention to the early portion of the distribution process, before reabsorption and redistribution of the isotope became a factor to confuse interpretation of the results.

From distribution studies in organs, a correlation became apparent between the richness in mitochondria of a given organ and its ability to concentrate manganese. We, therefore, extended our studies to include the mitochondrial uptake of this element *in vivo*, to determine whether these organelles were a preferential site of manganese accumulation and presumably of its metabolic activity.

Distribution of Manganese in Certain Organs

Methods—Mn^{56} (half life 2.59 hours) was prepared by thermal neutron bombardment (Mn^{55} (n^1, γ) Mn^{56}) of manganous sulfate powder[1] in the Brookhaven Nuclear Reactor. A dose of 100 μc., containing 38 γ of Mn^{++}

* This work was su ported by the Atomic Energy Commission. This paper was awarded an A. Cressy Morrison Prize by the New York Academy of Sciences, December 2, 1954.

[1] Spectrographic checks of the purity of our material were made through the courtesy of Morris Slavin, Department of Chemistry, Brookhaven National Laboratory. It was found necessary to use polyethylene bottles for liquid reagents.

in 0.5 ml. volume, was injected intraperitoneally into each of a series of eight Sherman rats weighing 200 ± 30 gm. One group of four animals was sacrificed by rapid exsanguination 15 minutes later, and another group of four after 1 hour. Selected organs were removed and weighed, and weighed portions placed in glass counting vials. The samples were counted in a well type scintillation counter (NaI (Tl) crystal), filtered to record only γ-radiation. Counting geometry was constant.[2] The counts of the organs were compared with the counts of a suitable dilution of the injected

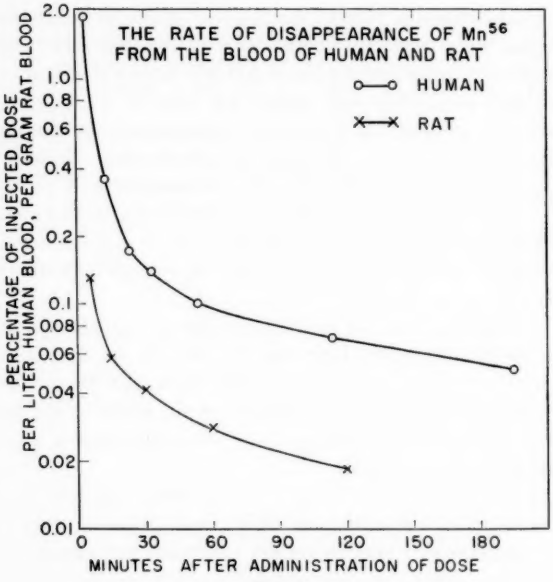

FIG. 1

dose after corrections for radioactive decay were made. The per cent of the injected dose taken up per gm. of tissue was calculated for each organ.

Results—The disappearance of manganese from the blood is both rapid and extensive, as can be seen in Fig. 1. The similarity of behavior observed in a human subject and in the rats suggested that manganese uptake in the human parallels that of the rat.

It will be noted (Table I) that liver, pancreas, and kidney have the highest concentrations of the isotope, while skeletal muscle and brain are

[2] Sample volumes from 0 to 2 ml. did not change the counting geometry. Determined through courtesy of E. E. Stickley, Division of Medical Physics, Department of Medicine, Brookhaven National Laboratory.

lowest. These tissues with the highest uptake of manganese have been reported to be rich in mitochondria (8, 9). The uptake of the isotope by these intracellular bodies was therefore studied.

Manganese Partition among Organelles of Rat Liver Cell

Methods—Six rats were injected intraperitoneally with Mn^{56} and with same dosage as in the study on organ distribution. They were sacrificed ½ hour later by decapitation. The livers were removed and weighed, and weighed portions of each were homogenized in cold 0.25 M sucrose. Fractionation was performed by the method of Schneider and Hogeboom (10). Radioactivity was determined on aliquots of the initial homogenate and of the "nuclear," mitochondrial, microsomal, and supernatant fractions. Total nitrogen was measured by the micro-Kjeldahl technique of Hiller, Plazin, and Van Slyke (11) as an estimate of the amount of tissue in each fraction.

TABLE I

Uptake of Mn^{56} by Various Organs

Per cent of dose per gm. of tissue.

Organ	Average weight	15 min. after injection		1 hr. after injection	
		Mean	Standard deviation	Mean	Standard deviation
	gm.				
Liver	8.56 ± 1.711	4.05	1.669	3.06	0.708
Pancreas	1.28 ± 0.229	3.30	0.614	2.70	1.109
Kidney	1.72 ± 0.256	1.23	0.331	1.43	0.329
Heart	0.74 ± 0.096	0.41	0.117	0.50	0.147
Thyroid	0.018 ± 0.0045	0.39	0.171	0.40	0.138
Bone	21.3* ± 2.88	0.15	0.034	0.22	0.054
Muscle	88.6* ± 12.05	0.062	0.029	0.070	0.017
Blood		0.058	0.019	0.028	0.011
Brain stem	0.45 ± 0.065	0.033	0.009	0.046	0.004
" hemispheres	1.06 ± 0.077	0.028	0.006	0.037	0.003

* Weights of these organs calculated from Donaldson (21).

To measure the loss of manganese from the particulate matter of the cell during the multiple washings required by the fractionation procedure, the liver homogenate from a rat injected with Mn^{56} was centrifuged at 80,000 × *g* for 45 minutes, and the supernatant fluid was removed and tested for radioactivity and nitrogen content. The sediment was washed and resedimented in fresh cold 0.25 M sucrose five times. Aliquots of each wash were analyzed for Mn^{56} and nitrogen content.

Conversely, it was necessary to test the possibility that a considerable part of the apparent mitochondrial uptake was due to passive adsorption of manganese from the solution onto the surface of the inactive mitochondria. To do this, freshly prepared mitochondria in cold 0.25 M sucrose were incubated from 20 minutes to 3 hours at room temperature with an Mn^{56} solution containing 0.5 μc. of Mn^{56} and 0.19 γ of Mn^{++} in a 10 ml. volume, and the removal of the isotope from the solution was measured after centrifuging the mitochondria.

Results—About three-fourths of the manganese radioactivity is found in the organelles of the cell, and of this the mitochondria contained about two-thirds (Table II). In terms of radioactivity per unit nitrogen, the

TABLE II

Uptake of Mn^{56} by Organelles of Rat

Fraction	Liver cell				
	Average N per ml. fraction	Uptake of fractions, per cent of whole liver uptake		Uptake of fractions per μmole tissue N (whole liver activity = 1.000)	
		Mean	Standard deviation	Mean	Standard deviation
	μmoles				
Nuclei	12.3 ± 3.4	16.4	3.57	0.95	0.164
Mitochondria	14.3 ± 2.7	41.3	15.02	1.97	0.564
Microsomes	14.3 ± 5.5	15.0	2.39	0.77	0.138
Supernatant	43.2 ± 13.1	28.9	6.55	0.54	0.155
Whole liver	65.4 ± 17.6	101.6		1.00	

mitochondria contained almost twice the concentration of manganese compared to whole liver, while in all the other fractions the concentration was lower than in the unfractionated homogenate. Statistical evaluation of these data, by the analysis of variance (12), revealed that a high degree of statistical significance can be attached to both sets of data. The probability of the observed variation among means occurring by chance is much less than 1 per cent. Furthermore, there was no significant departure from the observed intracellular distribution, even when the rats were sacrificed 6 minutes and 2 hours after the injection of the isotope, although the total liver content of manganese was altered.

Employing of the intracardiac route in place of the intraperitoneal changed neither uptake nor the intracellular partition of manganese.

That passive adsorption of the element onto the resting mitochondria was not a factor of importance is evident in that, on the average, less than 10 per cent of the Mn^{56} was removed from the solution by the mitochon-

dria *in vitro*, a negligible figure in comparison to the uptakes *in vivo* previously described (Table II).

Conversely, the loss of the isotope from the organelles into the supernatant fraction was readily apparent from a consideration of the amounts of Mn^{56} and of tissue nitrogen removed by each wash: If the Mn^{56}:N ratio of the initial supernatant fluid is expressed as 1.00, that of the succeeding five washes was 3.49, 3.79, 9.70, 7.12, and 11.51. It can be seen that an ever increasing proportion of manganese was removed with each succeeding wash.

These findings demonstrate that the intracellular distributions obtained by our methods represent minimal values for the particulate matter of the cells and maxima for the soluble fraction. Redistribution *in vitro* by adsorption of Mn^{56} on the mitochondria appears to be minimal.

DISCUSSION

The total amount of manganese administered to our animals was kept within physiological limits, on the order of 5 per cent of the daily dietary intake.[3] Thus, we avoided both toxic effects and the possibility of saturating an active transport mechanism and thereby altering the kinetics of the metal.

The rate of disappearance from the blood of this element forms a curve (Fig. 1) which can be graphically analyzed into three or more components. This is compatible with a multicompartmental system, one with high concentration and others with lesser concentrations of manganese, and led us to search for a site of high influx of this substance. From our organ distribution data, it was readily apparent that there was no obvious correlation between metabolic activity and manganese uptake (Table I) of the tissues studied. Furthermore, the high concentration in skeletal muscle of such manganese-activated enzymes as hexokinase, prolidase, isocitric dehydrogenase, phosphoglucomutase, and others (13, 14) was not reflected by its manganese uptake. On the other hand, the concentration of the element in mitochondria-rich organs, such as liver, pancreas, and kidney, was impressive. The low uptake of brain tissue is an apparent exception to this observation, which may be due to a blood brain barrier effect against the passage of metal. From these findings, it seemed quite probable to us that the mitochondria represented the site of high influx of manganese for which we were seeking. Our studies on liver cell fractionation (see Table II) indeed display an impressive accumulation of the isotope in these organelles. That the mitochondrial concentrations thus found are minimal and the supernatant concentrations maximal is demonstrated by the effect of repeatedly washing the former. Our data also

[3] Purina fox chow; analysis supplied by the manufacturer.

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demonstrate insignificant uptake of manganese by resting mitochondria *in vitro*. The nature of the transport mechanism for manganese is now being investigated.

In speculating about the function of manganese in the intracellular milieu, it is noteworthy, however, that the accumulation of manganese by the mitochondria to an extent far exceeding that of the other cellular components does not agree with our expectations from some of the available cytochemical data. To cite as an example an enzyme system active in protein metabolism and well studied by enzymologists, arginase, which requires manganese for activation, both *in vitro* (15) and *in vivo* (16), is not primarily a mitochondrial enzyme (17). In contrast, the most striking biochemical property of mitochondria has been shown to be their content of enzymes related to the respiratory activity of the cell (18). It seems quite probable that the concentration of manganese in the mitochondria is a reflection of its rôle as a respiratory cofactor. This view is supported by the work of Kenten and Mann on plant peroxidase systems (19), who found that the oxidation of *p*-cresol by peroxidase was accompanied by an oxidation of Mn^{++} to Mn^{+++}, with subsequent return to the Mn^{++} state. Recent additional confirmation for this hypothesis is found in studies by Lindberg and Ernster (20), which demonstrated marked stimulation of oxidative phosphorylation in isolated mitochondria with addition of manganous ions to the system.

SUMMARY

1. Intraperitoneally injected radioactive manganese (Mn^{56}) is rapidly distributed, concentrating primarily in organs rich in mitochondria.

2. Liver cell fractionation studies confirm the assumption that the mitochondria are the principal intracellular site of manganese uptake.

3. The observed distribution of manganese would be compatible with its functioning as a respiratory cofactor.

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REVERSAL OF ALCOHOL TOXICITY BY GLUTAMINE

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Growing evidence, recently summarized by Mardones (1), supports the conclusion that certain materials such as liver and yeast contain a nutritional factor, as yet unknown, which reduces the voluntary ethanol intake of experimental animals. Although several individual known vitamins of the B complex have been shown to produce this effect (2, 3), Mardones reported that in some animals a liver supplement induces a still further decrease in voluntary ethanol intake. Some desirable effects of nutritional supplements upon human alcoholism have been reported (4). In view of these results, and of the need for further information about the mechanism of alcohol toxicity and protection against it, a search was made for principles in liver extract and other natural materials which might prevent the inhibitory effect which ethanol and certain other alcohols exert upon growth of *Streptococcus faecalis*. The isolation of a liver factor which prevents the toxicity of alcohols for *S. faecalis* has been accomplished in the present investigation. The factor has been identified as glutamine.

EXPERIMENTAL

Methods—Cultures of *S. faecalis* (ATCC 8043) were maintained by conventional bacteriological practices. Cells for inoculation of assay medium were grown in yeast extract-peptone medium (5) for 20 to 24 hours at 30°, centrifuged, washed once with sterile 0.85 per cent sodium chloride solution, centrifuged, resuspended in 10 ml. of the saline, and diluted 1:50 with saline. Samples to be assayed were diluted with water to a volume of 5.0 ml. in 20 × 150 mm. lipless Pyrex tubes, which were then capped and sterilized by steaming for 15 minutes. The double strength medium was prepared and steamed separately for 15 minutes, cooled, and inoculated with 0.5 ml. of the dilute suspension of *S. faecalis* per 100 ml. of medium, and 5.0 ml. were added, with sterile pipettes, to each assay tube. The alcohol supplements were then added to each tube in the amounts shown in Tables I and II. For routine assays the alcohol supplement was added to the batch of sterilized medium before addition of the medium to the tubes.

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Assay Medium—Double strength basal medium containing acid-hydrolyzed casein, described previously (6), was supplemented with uridine (10 γ per 10 ml.) (7), Tween 80 (1 mg. per 10 ml.) (7), purines-pyrimidines and glucose as described previously (6), and vitamins as also described by Lansford and Shive in the same reference (6), with the exception that (*a*) the tabulated amounts of vitamins were dissolved in 30 instead of 100 ml. of water and (*b*) calcium pantothenate (3 mg. per 30 ml. of vitamin supplement) was included. Typical curves representing growth response of *S. faecalis* under these conditions to glutamine, and to hot water extract of liver, are shown in Fig. 1.

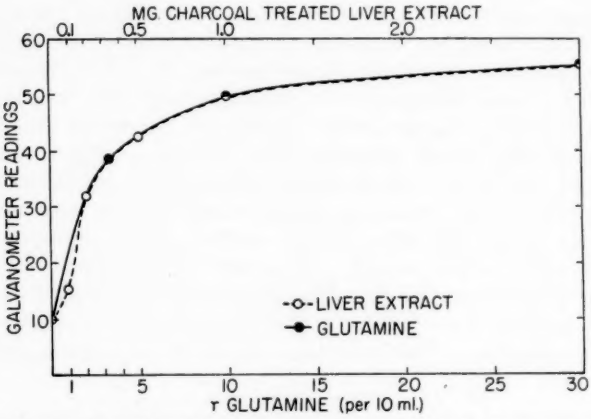

FIG. 1. Reversal of ethanol (0.3 ml. per 10 ml.) toxicity by liver extract or glutamine. Test organism, *S. faecalis*, incubated 18 hours at 30°.

Isolation Procedure. Preparation of Hot Water Extract of Liver—Fresh hog liver was cut into small pieces, mixed with an equal weight of water, and homogenized in a Waring blendor. The homogenate was steamed for 30 minutes and filtered; the residue was stirred into an equal weight of hot water and filtered, and the two filtrates combined. After determination of the dry solid content, Darco G-60 (weight equal to one-half the dry weight of liver solids present) was added, stirred 30 minutes, and removed by filtration. The charcoal-treated extract was used in the following concentration procedure.

Dowex 50 Column—Liver extract (750 ml.) containing 30 gm. of solids was allowed to run through a column composed of 40 gm. (air-dried) of Dowex 50 (hydrogen phase) resin suspended in water. After the column had been washed with 1 liter of water, the active principle was eluted with 50 per cent acetone-5 per cent concentrated ammonium hydroxide. The

yield of active principle, by assay with *S. faecalis*, was 50 per cent in the first 300 ml. of eluate, with about 3-fold purification of the activity (*i.e.*, 6-fold weight purification).

Super Filtrol Column—The above eluate (300 ml.) was evaporated to dryness, and the residue dissolved in 75 per cent ethanol. A portion of this solution, equivalent to 160 ml. of the Dowex 50 eluate and containing 2.66 gm. of solids, was passed through a column composed of 40 gm. of 200 mesh Super Filtrol suspended in 75 per cent ethanol. The column was next washed with 66 per cent ethanol, and the active principle then eluted with 50 per cent ethanol, with a 25 per cent over-all yield of activity, and 7.5-fold over-all purification.

Amberlite IRA-400 Column—The 50 per cent ethanol eluate from the Super Filtrol column was evaporated to dryness, and the residue was dissolved in a small amount of water. A portion of this solution equivalent to 3.6 gm. of the original 30 gm. of liver extract solids, and containing 120 mg. of solids, was passed through a column composed of 8 gm. (air-dried) of IRA-400 resin (chloride phase). The resin column was washed with water, and the active principle eluted with 1 per cent acetic acid (about 25 ml.), with a 20 per cent over-all yield of activity, and 16-fold over-all purification.

Alumina Column—The eluate from the resin column was evaporated to dryness, dissolved in a small volume of 66 per cent ethanol-water, and passed through a column composed of 10 gm. of activated alumina (Alcoa) suspended in 66 per cent ethanol. After the column had been washed with 66 per cent ethanol, the active principle was removed by elution with 60 ml. of 50 per cent ethanol, with a 20 per cent over-all yield of activity, and 64-fold over-all purification.

Identification As Glutamine—The material from the alumina column, when compared with L-glutamine by assay with *S. faecalis*, showed an apparent glutamine content of 65 per cent. More material of the same purity was obtained by processing the remainder of the Dowex 50 eluate through the same subsequent steps. Pure glutamine was then isolated by crystallizing the 65 per cent material from 80 per cent ethanol; the product was then recrystallized and characterized by its x-ray diffraction pattern, which was identical with that of L-glutamine.

Results

Under the assay conditions described above, various alcohols are inhibitory to the growth of *S. faecalis*, as shown in Table I. During the isolation work, either ethyl or *n*-propyl alcohol was employed in the assay medium; *n*-propyl alcohol appears slightly preferable since less volatilizes during incubation of the assay tubes.

While the assay was being employed for the isolation of the liver factor, various known compounds not already present in the medium were tested

TABLE I

Comparative Toxicities of Various Alcohols

Test organism, *S. faecalis* 8043; incubated 17 hours at 30°.

Alcohol	Galvanometer readings*							
	Methyl	Ethyl	*n*-Propyl	Isopropyl	*n*-Butyl†	*sec*-Butyl	*tert*-Butyl	*n*-Amyl†
ml. per 10 ml. tube								
0	77				64			
0.01								55
0.02								27
0.05		76	72	75	54	75	74	9
0.075					38			
0.1	80	70	54	68	13	60	66	
0.2	72	45	7	24		10	20	
0.3	49	8	4	6		5	8	
0.4	17			5			5	

* A measure of culture turbidity; distilled water reads 0, an opaque object 100.
† Separate experiment.

TABLE II

Reversal of Alcohol Inhibitions by Glutamine

Test organism, *S. faecalis* 8043; incubated 17 hours at 30°.

L-Glutamine	Galvanometer readings						
	Alcohol (amount shown per 10 ml. tube)						
	Ethyl, 0.3 ml.	*n*-Propyl, 0.2 ml.	Isopropyl, 0.3 ml.	*n*-Butyl, 0.1 ml.	*sec*-Butyl,* 0.15 ml.	*tert*-Butyl, 0.3 ml.	*n*-Amyl,* 0.05 ml.
γ per 10 ml.							
0	9	10	8	13	14	17	9
1	28						
2	42	31	26	32	30	15	19
5	50	43	32	39	40	20	35
10	56	52	48	50	53	26	39
20	71	56	57	51	53	36	31
50	78	64	57	59	65	53	47
100		72	71			49	53

* Separate experiment.

for ability to reverse this toxic effect of alcohols, and glutamine was found to be comparatively effective, as indicated by the data of Table II. In addition to the alcohols listed in Tables I and II, several other 5-carbon

alcohols were tested and found less inhibitory than *n*-amyl; their toxicity was also prevented by glutamine.

In order to establish that the liver principle which reversed alcohol toxicity is actually glutamine, the isolation procedure, presented above, was carried to completion. Applied to hot water extract of liver prepared as described above, the assay indicated that the liver extract solids are about 1 per cent as active as glutamine. Comparison of hot water extract of liver with other natural extracts by means of the *S. faecalis*-alcohol assay indicates that corn steep-liquor and some batches of cabbage juice are about equivalent to the liver extract in activity, per unit weight content; other batches of cabbage juice were 2 to 5 times as active. Liver extract E-1302 (Eli Lilly and Company) and yeast extract (Difco) are about one-half as active, and enzymatic casein hydrolysate twice as active, as the hot water liver extract described.

Attempts to carry out recovery experiments with the *S. faecalis*-alcohol assay upon liver and the natural extracts mentioned above have indicated the presence of other principles in these natural materials which exert a sparing effect upon the activity of known glutamine. Thus, the apparent 50 per cent loss in activity mentioned in the Dowex 50 isolation step may represent, not a loss of glutamine, but a separation of it from one or more other sparing principles.

SUMMARY

A factor which prevents the toxicity of alcohols for *Streptococcus faecalis* has been isolated from hot water extract of liver, and identified as glutamine.

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VOLUNTARY ALCOHOL CONSUMPTION BY RATS FOLLOWING ADMINISTRATION OF GLUTAMINE

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A number of investigators (1, 2) have postulated the presence of unknown factors in natural materials which will decrease the voluntary alcohol consumption of experimental animals. Since Ravel *et al.* (3) have demonstrated that glutamine is effective in reversing the inhibition of growth of *Streptococcus faecalis* caused by the inclusion of small amounts of alcohol in the medium, it seemed desirable to carry out a preliminary study to determine the effects of glutamine on the voluntary consumption of alcohol by rats. This paper is a report of that study.

EXPERIMENTAL

Thirty Wistar rats (designated as the H strain in previous communications from this laboratory), fifteen male and fifteen female, at 6 months of age were kept in separate cages in a temperature-controlled room. Attached to each cage were two bottles, one containing tap water and the other 10 per cent alcohol. The bottles were emptied, washed, and refilled, and the positions of the bottles alternated every 2 to 5 days. The animals were fed Purina laboratory chow throughout the experiment. In order that each animal might serve as its own control, the quantity of alcohol consumed by each animal on the control diet was measured every 2 to 5 days for a period of 55 days.

From this group nineteen rats, seven male and twelve female, were then selected on the basis of their higher alcohol consumption for glutamine administration. Each animal was fed daily 100 mg. of glutamine mixed with ground laboratory chow for a period of 9 days, and measurements were made of the alcohol consumed.

The nineteen rats were then divided at random into two groups. During the following 17 day period, one group (four males and six females) continued to receive 100 mg. of glutamine orally each day, while those in the other group (three males and six females) were injected each day intraperitoneally with 50 mg. of glutamine dissolved in 1 ml. of 0.85 per cent sodium carbonate solution. The rats receiving glutamine orally were injected with

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1 ml. of sodium carbonate solution. The alcohol consumed by each animal was measured as before.

TABLE I

Effect of Glutamine on Alcohol Consumption by Rats

Rat No.	Sex	Weight, gm.	Period before glutamine administration, 55 days	Period of glutamine administration			Period following glutamine administration, 63 days	Per cent change in alcohol consumption induced by glutamine administration
				1st 9 days, 100 mg. per rat per day, given orally	Next 17 days, 100 mg. per rat per day, given orally	Average consumption for 26 day period		
Average alcohol consumption (ml. per 100 gm. rat per day) for rats of Group I								
1	F.	233	0.84	0.14	0.21	0.18	0.69	−79
2	"	227	0.52	0.23	0.48	0.39	0.75	−25
4	"	201	0.89	1.02	0.92	0.95	1.05	+07
5	"	228	0.89	0.63	0.67	0.66	0.90	−26
6	"	203	0.32	0.40	0.32	0.35	0.52	+09
7	"	239	0.51	0.24	0.26	0.25	0.73	−51
17	M.	332	0.27	0.14	0.32	0.26	0.18	−04
18	"	388	0.41	0.06	0.06	0.06	0.21	−85
19	"	377	0.33	0.17	0.23	0.21	0.26	−36
27	"	393	0.27	0.05	0.13	0.12	0.21	−56
Mean.....			0.53	0.31	0.36	0.34	0.55	−35
Average alcohol consumption (ml. per 100 gm. rat per day) for rats of Group II								
8	F.	240	0.84	0.41	0.66*	0.58	0.30	−31
9	"	227	0.64	0.13	0.45*	0.33	0.89	−48
10	"	236	0.39	0.28	0.35*	0.33	0.74	−15
13	"	219	0.38	0.59	0.88*	0.78	0.98	+105
14	"	227	0.41	0.19	0.69*	0.51	0.60	+24
15	"	233	0.70	1.02	0.98*	0.99	0.89	+41
22	M.	363	0.34	0.37	0.38*	0.38	0.40	+12
24	"	408	0.42	0.12	0.05*	0.08	0.11	−81
30	"	365	0.38	0.15	0.32*	0.26	0.20	−32
Mean.....			0.50	0.36	0.53*	0.47	0.57	−03

* Glutamine (50 mg. per rat per day) intraperitoneally instead of orally.

At the end of the 17 day period, all the rats were returned to a pellet diet and their alcohol consumption was measured for an additional 63 days.

The average amount of alcohol consumed daily by each of the animals during each of the four periods is presented in Table I.

DISCUSSION

As can be seen from Table I, glutamine administered orally appears to be a relatively effective agent in decreasing the voluntary consumption of alcohol by rats. In the first group of rats the daily consumption was only 65 per cent as great during the 26 days of glutamine administration as during the previous control period. It is worth noting that, while one rat drank only 15 per cent as much and another only 21 per cent as much during the period, glutamine was almost wholly ineffective in reducing the alcohol consumption of three rats of this group. This is further evidence that in experimental animals individuality in metabolism exists (4), and is in confirmation of the genetotrophic concept (5, 6).

In this connection it may be pointed out that the nutritional status of the closely related compound glutamic acid has been in a state of confusion for a number of years. Investigators have repeatedly reported improvements in the intelligence level of mentally deficient children upon administration of glutamic acid, only to have other investigators report that no such improvement was observed. The fact that some mentally deficient individuals do show large gains as a result of glutamic acid therapy while others show no response at all probably is responsible, at least in part, for this confusion. Foale (7), for example, working with a group of mentally deficient children, found that the difference between the responses of the group on glutamic acid therapy and the control group was small; however, the "intelligence level" of some individuals on glutamic acid therapy showed fairly large gains (as much as eleven points), while none in the control group improved more than three points.

As with other nutritional supplements which have previously been found to reduce alcohol consumption in animals (8), the effect of glutamine does not persist long after its discontinuance. During the 63 day period following administration of glutamine, every animal in the first group except one returned to a higher rate of alcohol consumption than during the glutamine period.

The data on the second group of rats seem to indicate that glutamine is much less effective when injected than when fed orally. The animals of this group drank only 72 per cent as much alcohol when fed glutamine as during the control period, but when glutamine was injected the alcohol consumption returned to a level equivalent to that of the control period. The possibility of increased alcohol consumption due to a shock reaction from the injection was minimized by injecting the animals of the first group with an equivalent amount of sodium carbonate solution. The question arises as to whether glutamine must be transformed in the intestinal tract to some other agent in order to be effective.

Statistical analysis of the data presented in Table I reveals that, for all

nineteen rats taken as a group, the difference in mean alcohol consumption during the 9 day oral glutamine supplementation period as compared with the previous control period was significant at the 5 per cent confidence level ($P = 0.95$; $t = 2.15$). When the data were expressed as gm. of alcohol per rat per day and a similar statistical analysis was made, the difference was significant at the 1 per cent confidence level ($P = 0.99$; $t = 3.09$).

We now have similar experiments under way comparing the effects of glutamine, glutamic acid, and other nitrogen supplements on alcohol consumption. These results will be reported in a later paper.

SUMMARY

1. Glutamine fed at a level of 100 mg. daily caused a statistically significant lowering of the voluntary consumption of alcohol by rats.

2. Injected glutamine appeared to be relatively ineffective.

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STUDIES ON THE INCORPORATION OF C^{14} ADMINISTERED AS L-SORBOSE INTO L-ASCORBIC ACID AND D-GLUCOSE IN RATS*

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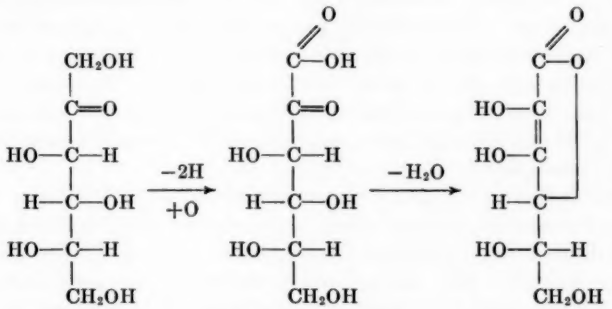
Previous studies on the biosynthesis of ascorbic acid have shown that glucose is converted to ascorbic acid in Chloretone-treated rats without prior cleavage of its carbon chain (1–3). L-Sorbose, a ketohexose like fructose, has been suggested as a possible intermediate in the biosynthesis of L-ascorbic acid from glucose (4). This L-sugar has a close structural relationship to L-ascorbic acid, to which it can be converted as shown in the accompanying scheme.



L-Sorbose — 2-Keto-L-gulonic acid — L-Ascorbic acid

To investigate this precursor relationship, a C^{14} tracer study has been carried out in Chloretone-treated rats with L-sorbose, labeled both uniformly in all 6 carbon atoms and specifically in carbon 6. The results of this investigation indicate that L-sorbose is not a direct precursor of L-ascorbic acid but undergoes fragmentation before being converted to

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L-ascorbic acid. Further information was obtained in this study on the biological fate of L-sorbose. This ketohexose is extensively metabolized to CO_2 and is converted to an appreciable extent to liver glycogen by a pathway which involves a prior cleavage of its carbon chain.

EXPERIMENTAL

Radioactive Material—L-Sorbose-C_6^{14} and L-sorbose-6-C^{14} were prepared from glucose-C_6^{14} and glucose-1-C^{14}, respectively, by catalytic hydrogenation, followed by oxidation of the resulting D-sorbitol with *Acetobacter suboxydans* (5). Glucose-C_6^{14} was prepared from $C^{14}O_2$ by photosynthesis and glucose-1-C^{14} was obtained from the National Bureau of Standards, Washington, D. C. The specific activities of L-sorbose-C_6^{14}, L-sorbose-6-C^{14}, and D-glucose-C_6^{14} used in the animal experiments were 0.25, 0.23, and 0.47 μc. per mg., respectively.

The chemical and radioactive purity of the labeled L-sorbose was established as follows: The labeled L-sorbose melted at 162–164°, which is identical to that of a sample of authentic L-sorbose. Mixed melting points of the labeled and authentic compounds showed no depression. No change in the specific activities of the labeled compounds was detected on three successive crystallizations from an ethanol-H_2O mixture. Identical R_F values were obtained when the labeled and authentic compounds were submitted to paper chromatography with the solvent system *n*-butanol-acetic acid-water (3:1:3, volume per volume) (6). A radioautograph of the paper chromatogram yielded a single spot. The labeled L-sorbose was also subjected to ion exchange chromatography as its borate complex (7).[1] The chromatograms further confirmed the radioactive homogeneity of the labeled compound, since only one peak, identical to that of L-sorbose, was obtained.

To preclude any possibility of randomization of the C^{14} during the oxidation of D-sorbitol with *A. suboxydans*, L-sorbose-6-C^{14} was subjected to partial degradation. This labeled compound was converted to its phenylosazone derivative and treated with periodate (8) to yield carbon 6 as formaldehyde. All the radioactivity of the original compound was found in formaldehyde isolated as its dimedon derivative, indicating that the C^{14} in the L-sorbose molecule resided completely in carbon 6.

Biological Tests—Studies on the conversion of L-sorbose to L-ascorbic acid were carried out in essentially the same manner as those previously described on the precursor relationship between glucose and ascorbic acid (1): Male albino rats of the Wistar strain, weighing 250 to 300 gm., were maintained on a basal diet of evaporated milk for 2 weeks before each ex-

[1] The ion exchange chromatograph of L-sorbose was carried out through the courtesy of Dr. L. P. Zill, Biology Division, Oak Ridge National Laboratory.

perimental period. 45 mg. of Chloretone were administered daily for a period of at least 4 days prior to the injection of the labeled compound. The Chloretone was given by stomach tube as a homogenate in 1 ml. of evaporated milk. 24 hour urine samples were collected in 5 ml. of 10 per cent oxalic acid. Urinary excretion of ascorbic acid was determined by titration with indophenol dye. During the control period the animals excreted about 1 mg. of ascorbic acid daily, which increased to an average of 30 mg. of ascorbic acid daily 4 days after Chloretone administration. Weighed quantities (10 to 20 mg.) of the labeled test substances in 1 ml. of water were administered by intraperitoneal injection. The animals were then placed in a metabolism chamber (1) and respiratory CO_2 was collected at various intervals up to 24 hours. The ascorbic acid was isolated from the urine after the addition of 150 mg. of carrier ascorbic acid by an ion exchange method (1). A portion of the ascorbic acid was isolated as its 2,4-dinitrophenylosazone derivative. The radioactive purity of the isolated osazone was established by the same method as used previously (1). No change was found in the specific activity of the osazone after successive recrystallizations, and the ascorbic acid isolated from the same urine samples had a molar specific activity identical with that of its osazone derivative. L-Sorbose was isolated from the urine as its phenylosazone derivative after addition of 300 mg. of carrier L-sorbose.

The studies on the conversion of L-sorbose to liver glycogen were carried out as follows: Male albino rats weighing 250 to 300 gm., which were fasted for 24 hours, were given by stomach tube 4.0 mmoles of non-radioactive glucose per 100 gm. of body weight and immediately afterwards weighed quantities (5 to 10 mg.) of the labeled compounds were administered by intraperitoneal injection. The respiratory CO_2 was collected for 3 hours, at the end of which time the animals were sacrificed and their livers removed. Liver glycogen was isolated and hydrolyzed to glucose, and the amount of resulting glucose was assayed (8). Carrier glucose (1 gm.) was added and the labeled glucose used for degradation studies was obtained after crystallization with absolute ethanol.

Degradation Procedures—Glucose of the liver glycogen was treated with 10 per cent hydrobromic acid to yield carbon 1 as formic acid and carbons 2 to 6 as levulinic acid (9). The acids were separated from the reaction mixture by continuous ether extraction and isolated as the sodium salts by column partition chromatography with dilute sulfuric acid (0.5 N) as the stationary phase (10). Levulinic acid was eluted with chloroform and formic acid with 10 per cent butanol in chloroform. Carbon 6 was obtained independently as formaldehyde by periodic acid degradation (11).

L-Ascorbic acid isolated from the urine was degraded as follows: Carbon 1 was obtained by decarboxylation of L-ascorbic acid with hot mineral

acid and carbon 6 was obtained as formaldehyde by periodate degradation of the 2,4-dinitrophenylosazone of ascorbic acid (2).

Radioactivity Determinations—Preparation of samples and assay for C^{14} were carried out as described previously (12).

Results

The amounts of C^{14} excreted in CO_2 and urine and incorporated in urinary ascorbic acid during a 24 hour period after administration of L-sorbose-6-C^{14}, L-sorbose-C_6^{14}, and D-glucose-C_6^{14} to Chloretone-treated rats are shown in Table I. An average of about 50 per cent of the administered C^{14} was found in the respiratory CO_2 during the 24 hour period after injection of the two differently labeled L-sorbose tracers, indicating that this L-sugar is appreciably metabolized in the rat. An average of 25 per cent of the C^{14} administered as L-sorbose is excreted in the urine. About 70 per cent of the total urinary C^{14} was shown to be present as L-sorbose by isolation of its osazone derivative.

TABLE I

C^{14} in Respiratory CO_2, Urine, and Urinary Ascorbic Acid during 24 Hour Period after Administration of L-Sorbose-C_6^{14}, L-Sorbose-6-C^{14}, and D-Glucose-C_6^{14} to Chloretone-Treated Rats

Substance administered	Experiment No.	Per cent of dose in		
		CO_2	Urine	Urinary ascorbic acid
L-Sorbose-C_6^{14}	R-38	61.4	30.8	0.43
"	R-40	57.0	15.8	0.27
L-Sorbose-6-C^{14}	R-91	46.8	31.2	0.26
"	R-94	37.2		0.32
D-Glucose-C_6^{14}	W-3*	79.4	3.05	0.27
"	W-4*	79.3	4.37	0.30

* These results were obtained in previously published experiments (1).

About 0.3 per cent of the dose of C^{14} administered either as L-sorbose-C_6^{14} or L-sorbose-6-C^{14} was found in the urinary L-ascorbic acid, which is about the same incorporation of C^{14} as found after administration of D-glucose-C_6^{14} (Table I). It is not possible to make any conclusions on the precursor relationship of L-sorbose for L-ascorbic acid based on values for the fraction of the dose of C^{14} incorporated into urinary ascorbic acid. For instance, glucose was found to be converted to L-ascorbic acid directly without cleavage of its 6-carbon chain (1–3), despite the small fraction of C^{14} incorporated in the L-ascorbic acid following administration of isotopic glu-

cose. If L-sorbose was converted directly to L-ascorbic acid, it would be expected that the major part of the C^{14} in the urinary L-ascorbic acid would be in carbon 6 following administration of L-sorbose-6-C^{14}. The L-ascorbic acid isolated from the urine of rats receiving L-sorbose-6-C^{14} was subjected to a degradation procedure in which the amount of C^{14} present in carbon 1 and carbon 6 was determined (Table II). About one-half of the total C^{14} present in L-ascorbic acid was distributed equally between carbon 1 and carbon 6 and the remainder in carbons 2 to 5, indicating that L-sorbose is not converted directly to L-ascorbic acid but only after cleavage of its carbon chain.

The incorporation of C^{14} in respiratory CO_2 and glycogen during a 3 hour period after administration of L-sorbose-C^{14}_6, L-sorbose-6-C^{14}, and D-glucose-C^{14}_6 to fasted rats was determined (Table III). The results indi-

TABLE II

Distribution of C^{14} in Urinary Ascorbic Acid after Dose of L-Sorbose-6-C^{14} to Chloretone-Treated Rats

Experiment No.	Per cent total C^{14} in ascorbic acid		
	Carbon 1	Carbon 6	Carbon 2-5*
R-94	24.2	24.0	51.8
R-95	28.8	29.5	41.7

* C^{14} in carbons 2 to 5 obtained by difference.

cate that L-sorbose is converted to glycogen to an appreciable extent; in fact, more C^{14} was present in glycogen after L-sorbose administration than after glucose administration under the same conditions. It will be noted that the same amount of incorporation of C^{14} in liver glycogen was obtained following administration of the two differently labeled L-sorbose tracers, indicating that the carbon 6 was converted to glycogen to the same extent as the average of all 6 carbon atoms.

The high degree of incorporation of isotopic L-sorbose into glycogen might suggest that L-sorbose is converted directly into glucose of liver glycogen by a pathway which does not involve a breakdown of its carbon chain. However, if this was so, one would expect that glucose of liver glycogen would be labeled predominantly on carbon 1 following the administration of L-sorbose-6-C^{14}, since a complete inversion of the molecule would have to occur to convert the L-sugar to one of D configuration. Glucose isolated after hydrolysis of glycogen from rats receiving L-sorbose-6-C^{14} was subjected to a degradation procedure in which the amount of C^{14} present in carbon 1, carbons 2 to 5, and carbon 6 was determined

(Table IV). It was found that the average values for the per cent of total C^{14} in glucose present in carbons 1 and 6 were 43 and 35 per cent, respectively; the remaining 22 per cent of C^{14} in glucose was in carbons 2 to 5. These findings indicate that L-sorbose is not converted to glucose directly, but proceeds by a pathway involving a fragmentation of its carbon chain.

TABLE III

Incorporation of C^{14} in Respiratory CO_2 and Glycogen during 3 Hours after Administration of L-Sorbose-C^{14}_6, L-Sorbose-6-C^{14}, and D-Glucose-C^{14}_6 to Fasted Rats

Substance administered*	Experiment No.	Weight of glycogen	Per cent of dose	
			CO_2	Glycogen
		mg.		
L-Sorbose-C^{14}_6	R-42	158	24.0	11.6
"	R-43	50	25.6	11.6
L-Sorbose-6-C^{14}	S-1	111	14.0	12.5
"	S-2	141	14.4	10.5
"	S-4	176		12.0
D-Glucose-C^{14}_6	R-45	157	26.8	7.1
"	R-46	128	25.1	8.8

* The rats received by stomach tube 4.0 mmoles of non-radioactive glucose per 100 gm. of body weight and immediately thereafter a weighed quantity (5 to 10 mg.) of the labeled compound was administered by intraperitoneal injection.

TABLE IV

Distribution of C^{14} in Glucose of Liver Glycogen after Dose of L-Sorbose-6-C^{14} to Fasted Rats

Experiment No.	Per cent total C^{14} in glucose			
	Carbon 1	Carbon 6	Carbons 2-6	Carbons 2-5*
S-2	42.2	31.6	57.5	26.2
S-4	44.5	37.2	55.8	18.3

* C^{14} in carbons 2 to 5 obtained by difference of C^{14} in carbons 1 and 6 from total C^{14} in glucose.

DISCUSSION

The finding that L-sorbose is not directly converted to L-ascorbic acid but only after splitting of the hexose chain furnishes strong evidence that the ketose is not an intermediate in the biosynthesis of L-ascorbic acid from glucose. Since L-sorbose undergoes fragmentation to yield glucose, the conversion of L-sorbose to L-ascorbic acid may, in fact, proceed via glucose as an intermediate. Failure to demonstrate a precursor relationship between L-sorbose and L-ascorbic acid is in agreement with the results of

Isherwood *et al.* (4) who reported no increase in the biosynthesis of L-ascorbic acid as detected by chemical analysis, when cress seedlings were grown in the presence of L-sorbose or when this sugar was injected into rats. The most likely pathway for the biosynthesis of L-ascorbic acid from glucose would seem to be via the intermediate formation of D-glucuronolactone. This mechanism has been suggested based on studies with C^{14}-labeled glucuronolactone in Chloretone-treated rats (13) and non-isotopic experiments with rats and cress seedlings (4).

The present study indicates that the conversion of L-sorbose to glucose proceeds via a pathway which involves fragmentation of the hexose chain of L-sorbose. The initial step in the metabolism of this ketohexose is very likely the formation of L-sorbose-1-phosphate; such a phosphorylation step has been shown to be catalyzed by fructokinase *in vitro* (14). A possible scheme for the conversion of L-sorbose-1-phosphate to glucose is suggested from some recent enzymatic studies, indicating that fructose-1-phosphate is converted to glucose via the intermediate formation of D-glyceraldehyde and dihydroxyacetone phosphate (15). In a similar fashion, L-sorbose-1-phosphate may be broken down to L-glyceraldehyde and dihydroxyacetone phosphate; the reverse step, the condensation of L-glyceraldehyde with dihydroxyacetone phosphate to form L-sorbose-1-phosphate, is catalyzed by aldolase (16). L-Glyceraldehyde, after being phosphorylated, presumably would be isomerized to D-glyceraldehyde phosphate via the intermediate formation of dihydroxyacetone phosphate. D-Glyceraldehyde and dihydroxyacetone phosphate would be converted to glucose via fructose-1,6-diphosphate (15). This scheme would explain the conversion of L-sorbose-6-C^{14} to D-glucose labeled predominantly in carbon 1 and carbon 6. The finding that L-sorbose is converted to glucose via triose intermediates would suggest that an important pathway for conversion of fructose to glucose in the intact animal may also be via formation of trioses.

SUMMARY

L-Sorbose has been synthesized labeled with C^{14} both uniformly in all 6 carbon atoms and specifically in carbon 6. Studies with these labeled compounds have shown that L-sorbose is not a direct precursor of L-ascorbic acid in Chloretone-treated rats, since its carbon chain undergoes fragmentation before being converted to L-ascorbic acid. In addition, it was found that L-sorbose is extensively metabolized to CO_2 and is converted to glucose of the liver glycogen by a pathway which involves fragmentation of its carbon chain. A scheme is presented for the conversion of L-sorbose to glucose via the intermediate formation of L-glyceraldehyde and dihydroxyacetone phosphate.

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INCORPORATION OF LABELED LEUCINE INTO CAROTENE BY PHYCOMYCES

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In studies of carotene production by *Phycomyces blakesleeanus* at Berkeley, yeast autolysate has been used to insure rapid and vigorous growth (1). When C^{14}-labeled sugar was incorporated into the medium, it appeared that the organism preferentially selected the non-sugar carbon for incorporation into the carotene molecule (2). Yeast autolysate was therefore clearly undesirable in any experiment designed to test the degree to which the carbon of an amino acid such as leucine might be incorporated.

A preliminary trial showed that a medium which at Liverpool (3) yielded satisfactory growth of *P. blakesleeanus* was inadequate for the Berkeley cultures; sparse growth developed slowly over a 2 week period of observation. We then tested Difco carbon base, 3 per cent (Wickerham),[1] essentially glucose plus known vitamins and trace elements, supplemented by 0.3 per cent asparagine, or 0.15 per cent asparagine and 0.15 per cent leucine, and this gave very satisfactory results.

Tests indicated heavy growth in 5 days for the medium with asparagine only, with a dull gray cast, and somewhat less growth in the same time for the asparagine-leucine medium, but here the culture was bright yellow in color. These media were used to test C-1- (carboxyl) and C-2-labeled leucine to determine the degree to which these carbons might be incorporated into the carotene.

EXPERIMENTAL

The DL-leucine-1-C^{14} and DL-leucine-2-C^{14} were prepared by chemical synthesis at the Donner Laboratory,[2] and incorporated with inactive

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[1] The Difco Bacto-yeast carbon base is fully defined by Wickerham ((4) Table I, Column 4): "Carbon base for nitrate assimilation test." It is comprised of glucose, salts, trace elements, vitamins, and trace amounts of the amino acids, L-histidine, DL-methionine, DL-tryptophan.

[2] We are indebted to Dr. B. M. Tolbert and Dr. R. M. Lemmon for the loan of these compounds.

leucine at a final concentration of 0.15 per cent leucine into solutions containing 3 per cent Difco carbon base and 0.15 per cent asparagine.

Samples of each medium were dried and burned in duplicate, and the CO_2 collected and converted to $BaCO_3$. Counts were as follows: medium containing DL-leucine-1-C^{14}, 1245, 1317 c.p.m. per mg.; medium containing DL-leucine-2-C^{14}, 1090, 960 c.p.m. per mg.

It may of course be argued that the effective counts, representing the L isomer only, should be 640 and 513 respectively. It is of interest, therefore, to record the specific activity of the CO_2 evolved from the DL-leucine-1-C^{14} after the spores had germinated.

TABLE I

Effect of Ionone on Synthetic Media

Time of growth, 5 days.

	Dry weight	Carotene	
		Total	
	gm.	*γ*	*γ per gm.*
0.15% asparagine-0.15% leucine			
Control	0.600	410	683
+ ionone*	0.392	1125	2870
0.3% asparagine			
Control	0.758	121	160
+ ionone*	0.704	1780	2530

* 20 μl. per plate.

Cultures containing DL-leucine-1-C^{14}, twenty plates, were placed in an air-tight box with a transparent Lucite cover, and CO_2-free air was slowly drawn through. The CO_2 liberated was trapped in sodium hydroxide and precipitated as $BaCO_3$. The activity rose, from 299 c.p.m. per mg. on the 3rd day, to 812 on the 4th, 1187 on the 5th, and 1697 on the 6th, when the mats were harvested.

On the 6th day after inoculation, the cultures were harvested. As these were active, the mats were immediately rinsed with water, with alcohol, and then extracted. In both cases, which differed from previous experience with mats grown on yeast autolysate, saponification was necessary before the samples could be chromatographed. Each was refluxed for 6 minutes with an equal volume of 20 per cent KOH in methanol, then washed with water, dried with Na_2SO_4, and chromatographed on a column of magnesia. After elution of the phytofluene, the β-carotene was removed from the column with petroleum ether containing 3 per cent acetone and crystallized. From the twenty plates in each set, sufficient carotene was ob-

tained (about 3 mg.) so that inert carrier was not needed. The crystalline product was washed and dried as previously described (2), samples were then burned in duplicate, and the resulting CO_2 was converted to $BaCO_3$ and counted. The results are as follows: DL-leucine-1-C^{14}, 60 and 86 c.p.m. per mg.; DL-leucine-2-C^{14}, 262 and 276 c.p.m. per mg.

These results show, as might be anticipated, that the carboxyl carbon of leucine was not incorporated to any appreciable extent. The carbon atom 2 of leucine is incorporated to a considerably greater extent. However, allowing for dilution of activity by the carotene synthesized from non-leucine carbon, one cannot explain the increase (see Table I) in carotene synthesis in the presence of leucine on the assumption of a selective use of the residual 5-carbon decarboxylated fragment. This does not rule out the incorporation of smaller fragments not involving the carbon atom 2.

We decided also to test the ionone effect on carotene synthesis in asparagine- and leucine-containing media in the presence of the Difco carbon base. Cultures were grown as before; procedures were identical with those previously described, except for the inclusion of the step with saponification.

The results are shown in Table I. It is to be remarked that the ionone-treated cultures were the most deeply colored of any yet grown in the Berkeley laboratory.

SUMMARY

It is shown that the mold *Phycomyces blakesleeanus* decarboxylates leucine, and the CO_2 resulting therefrom is not incorporated into carotene to any appreciable extent. Furthermore, when grown on Difco carbon base, the mold utilized the residual 5-carbon fragment of the decarboxylated leucine in synthesizing carotene only to a limited extent.

In the presence of ionone, both leucine- and asparagine-containing cultures yield high concentrations of β-carotene.

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HETEROGENEITY OF INTRACELLULAR RIBONUCLEIC ACID IN RAT LIVER CELLS*

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The intracellular distribution of ribonucleic acid (RNA) in rat liver cells has been repeatedly reported by several investigators, and reference is made to a review of the subject (1). The microsomal fraction was found to be preferentially rich in RNA, whereas in mitochondria the amount was so low as to be of questionable significance. The possible relation of the structure of RNA to the synthesis of differentiated proteins (2–4) has prompted us to investigate the composition of RNA isolated from various cellular fractions. Some fragmentary evidence existed in the literature to support the idea that the nucleotide composition of particulate RNA is characteristic of each cellular organelle (5–7), whereas other data did not support such a view (8).

The present investigation was undertaken to establish quantitatively the nucleotide composition of RNA prepared from various cellular fractions isolated by differential centrifugation. The results show that the RNA composition is characteristic of the structural units of the cell.

Methods

Preparations of Homogenate and Cellular Fractions—Animals of an average weight of 300 gm. (Wistar rats) were stunned and immediately decapitated. The liver was perfused with 50 ml. of 0.25 M sucrose by way of the portal vein. The whole liver was excised, placed on cracked ice, chilled for 2 minutes, and rolled on filter paper to sponge off the sucrose solution. In the first series, a pool of four adult rat livers was used and, in the second series, a pool of three. The pooled organs were immediately minced in a cold Plexiglas squeezer, and the pulp obtained was divided into three portions: (1) 3 gm. were used for the isolation of the total tissue RNA; (2) 6 gm. were homogenized in 0.25 M sucrose and subjected to differential centrifugation, and the total RNA of each cellular particulate was isolated; (3) 3 gm. were concurrently homogenized and centrifuged as above for

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the purpose of quantitative microscopic analysis of the fractions. Homogenization was carried out with a glass homogenizer during an interval of time sufficient for maximal cell breakage. The final volume was adjusted to give a 10 per cent homogenate. Four cellular fractions, namely nuclear, mitochondrial, microsomal, and supernatant fractions, were isolated by differential centrifugation according to the method of Schneider and Hogeboom (9) with a Servall SS-1 and a Spinco (model L) refrigerated centrifuge.

Isolation of RNA and Chromatographic Analysis—The nucleic acids were isolated from homogenate and cellular fractions by a slightly modified (10) Schmidt-Thannhauser procedure (11). After extraction of acid-soluble compounds and phospholipides from tissue pulp or cellular fraction, the residual material was dried and stored at 5°. The dry powder was hydrolyzed in 1.5 N KOH during 42 hours at 37° when needed for analysis. In one experiment, the dry powder was hydrolyzed with 0.3 N KOH during 24 hours at 37° to compare with the conditions described above. No difference was observed in the proportion of the nucleotides released from RNA in both conditions (Table I). On the other hand, the yield of free nucleotides was much higher when hydrolysis was performed in the presence of 1.5 N KOH during 42 hours at 37°. When the dry powder was hydrolyzed in 0.3 N KOH, the mononucleotides yield amounted to only 70 per cent of the optical density of the original hydrolysate, whereas with 1.5 N KOH the yield was to 98 per cent. With 1 N KOH, the extent of the hydrolysis was 90 per cent, which is comparable to the data of Smith and Allen (12). 1.5 N KOH was therefore preferred because hydrolysis was more nearly complete. This is of practical importance in dealing with cellular fractions of low RNA content.

At the end of the hydrolysis period, the incubated mixture was centrifuged. The small amount of insoluble material was washed three times with water. The supernatant fluid and washings were pooled and adjusted to a final volume of 30 ml. with glass-distilled water. This solution of nucleotides was then introduced by gravity flow in a column (13 cm. × 0.78 cm.) filled with resin Dowex 1. The column was then washed with glass-distilled water until the eluent reached pH 6.0 to 6.5. Elution was carried out with hydrochloric acid solutions at various concentrations (13). The elution was followed by measuring the optical density, at 260 mμ, of each fraction collected. A Beckman model DU spectrophotometer was employed. The concentration of each fraction was obtained from standard curves and expressed in micrograms of nucleotide per ml. of eluent. The standard curves were obtained by measuring the optical density at 260 mμ of different dilutions of each nucleotide (commercial, Schwarz Laboratories) in hydrochloric acid solutions at the same normalities that

were used for elution (13). The extinction coefficients[1] of these nucleotides, in acid and alkaline media, were essentially similar to those reported by Volkin and Cohn (14).

RESULTS AND DISCUSSION

Purity of Isolated Cellular Fractions—The quantitative examination of each cellular fraction by phase microscopy in a Petroff-Hausser bacteria counter was performed as previously described (15). The nuclear fraction contained 15 per cent of mitochondria, whereas the microsomal fraction appeared free of mitochondria. The supernatant fluid was optically free of particles. The mitochondrial fraction appeared uncontaminated by microsomes.

Correction for the mitochondrial contamination of the nuclear fraction, based on the number of mitochondria present in these fractions and on the absolute amount of each nucleotide released from their respective RNAs, can be calculated by simple mathematical means. Table II shows the differences between uncorrected and corrected nucleotide proportions of the nuclear RNA. These differences are smaller than those noted between the two groups of rats under observation. The contamination of the nuclear fraction by mitochondria is apparently insignificant so far as the RNA nucleotide composition is concerned.

RNA Composition of Normal Rat Liver Homogenate—Table I shows the nucleotide composition of RNA in moles per hundred moles of nucleotide. The homogenate RNA contained predominantly guanylic (34.3 moles) and cytidylic (27.1 moles) acids. Uridylic and adenylic acids were found in the same concentrations (19.5 and 19.1 moles).

Table I indicates also that the proportion of each nucleotide released from RNA was similar after strong (1.5 N KOH) or mild (0.3 N KOH) alkaline hydrolysis. Although it has been shown that pure cytidylic acid is deaminated in strong alkaline solution at 37° (16), conversion of cytidylic acid to uridylic acid does not proceed at an appreciable rate during hydrolysis of RNA in 1.5 N KOH compared to 0.3 N KOH.

RNA Composition of Cellular Fractions of Rat Liver—Table II shows the nucleotide composition of RNA in moles per hundred moles of nucleotides for the nuclear, mitochondrial, and microsomal fractions and the supernatant fluid. The RNA isolated from the nuclear and the microsomal fractions contained more guanylic acid (30.0 and 34.6 moles) than the RNA from the mitochondrial fraction and from the supernatant fluid (25.8 and 22.7 moles). Furthermore, the nuclear RNA differed from the microsomal RNA by higher content of uridylic and adenylic acids and lower content of

[1] The following extinction coefficients, at pH 2, were used: adenylic acid 14.3, guanylic acid 11.0, uridylic acid 10.0, and cytidylic acid 7.0×10^{-3}.

cytidylic acid. The mitochondrial RNA, on the other hand, differed from the supernatant fluid RNA by a slightly higher cytidylic acid and a much lower adenylic acid concentration. Only the supernatant fluid RNA contained an amount of uridylic and adenylic acids greater than that of cytidylic and guanylic acids. Table II thus demonstrates that the nucleotide composition of the RNA isolated from each of the various cellular fractions differs.

The difference in the RNA composition of each cellular fraction was also evident when the ratio of the different nucleotides to guanylic acid was calculated. The uridylic-guanylic acid ratios were, respectively, 0.83, 1.03, 0.64, 1.25 for nuclear mitochondrial and microsomal fractions and the supernatant fluid. A variation of the same magnitude was observed

TABLE I

Nucleotide Composition of Normal Rat Liver RNA Subjected to Various Conditions of Alkaline Hydrolysis

The results are expressed in moles per hundred moles of nucleotide.

Acid	Conditions of hydrolysis			
	1.5 N KOH, 42 hrs., 37°			0.3 N KOH, 24 hrs., 37°
	1st assay	2nd assay	Mean	1st assay
Cytidylic	29.3	24.9	27.1	26.9
Uridylic	19.5	19.5	19.5	18.9
Adenylic	18.6	19.6	19.1	17.7
Guanylic	32.6	36.0	34.3	36.5

for the adenylic-guanylic acid ratio. Consequently the over-all picture indicates that the supernatant fluid, mitochondrial, and nuclear RNA would contain 100, 60, and 30 per cent more uridylic and adenylic acids than the microsomal RNA on the basis of identical guanylic acid content. The ratio of cytidylic to guanylic acid varied to a much lesser degree. The supernatant fluid, mitochondrial, and nuclear RNA differed only by 34, 25, and 6 per cent from the microsomal RNA. It is concluded from these facts that each cellular fraction contains a specific RNA.

The composition of the homogenate and microsomal RNA, reported here, is similar to the results of Davidson and Smellie (8), but apparently discrepancies exist for the composition of the mitochondrial RNA. On the other hand, the RNA composition, described here, differs from that reported by Elson and Chargaff (5). Nevertheless the same conclusion is reached that the nuclear RNA and mitochondrial RNA are different from the RNA of the microsomal fraction and supernatant fluid. Furthermore,

the present results show that the microsomal and supernatant fluid RNAs are also different from each other.

Elson and Chargaff (17) have recently suggested regularity in the composition of cytoplasmic RNA, because the ratios of adenylic to uridylic acid, guanylic to cytidylic acid, and purines to pyrimidines were close to unity. In agreement with the present results, other analyses of RNA from cytoplasmic fractions (5, 8) have shown that these ratios differed from unity.

TABLE II

Nucleotide Composition of RNA Isolated from Cell Fractions of Normal Rat Liver

The results are expressed in moles per hundred moles of nucleotide.

		Cytidylic acid	Uridylic acid	Adenylic acid	Guanylic acid
Nuclei*	1st assay	22.2	24.8	24.3	28.7
	2nd "	20.4	25.1	23.2	31.3
	Mean	21.3	25.0	23.8	30.0
Mitochondria	1st assay	22.0	28.7	24.1	25.2
	2nd "	22.0	27.0	24.7	26.3
	Mean	22.0	27.9	24.4	25.8
Microsomes	1st assay	24.6	21.3	20.1	34.0
	2nd "	21.9	22.6	20.3	35.2
	Mean	23.3	22.0	20.2	34.6
Supernatant fluid	1st assay	19.1	29.0	27.4	24.5
	2nd "	21.6	27.8	29.7	20.9
	Mean	20.4	28.4	28.6	22.7

* The nucleotide composition of the nuclear fraction RNA, when corrected for the number of mitochondria present in this fraction and according to the composition of the mitochondrial RNA, gives the respective corrected values for the two assays: 22.2 and 20.1 for cytidylic acid, 24.4 and 24.7 for uridylic acid, 24.3 and 22.9 for adenylic acid, 29.1 and 32.3 for guanylic acid.

This study further supports the view that cellular fractions are chemical as well as morphological entities. While nuclei, mitochondria, microsomes, and soluble fractions were shown to differ in regard to their enzymatic properties (1), turnover, and composition of some of their components (1, 18–20), the present work revealed that they also differ in the composition of their RNAs.

In the light of these facts, it might be necessary to reexamine the problem of the origin of the cellular particulates and of their constituents. If the RNA of a given fraction is the precursor of the RNA in other fractions (7, 18, 19, 21), this possibility would imply, on the basis of our results,

important changes in the composition of the RNA during its transfer from one particle to another. The present facts are in line with the conclusion of Jeener (22) that the RNA of each fraction is synthesized *in situ*. The RNA would appear to be independently synthesized in each particulate according to the typical composition of the RNA already present.

SUMMARY

The nucleotide composition of the RNA of the homogenate and of the isolated cellular fractions of normal rat liver has been determined. It has been found that the RNA composition of each cellular fraction differs from the others and is specific for the fraction from which it has been isolated. This is discussed in relation to the origin of the RNA in various morphological units of the cell.

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ORIGIN OF GLUCOSE AND GLYCOGEN CARBONS FORMED FROM C^{14}-LABELED PYRUVATE BY LIVERS OF NORMAL AND DIABETIC RATS*

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The availability of carbon-labeled substrates and of methods for studying carbohydrate metabolism in muscle and in liver *in vitro* has made it possible to study quantitatively some of the detailed reactions involved in carbohydrate production and utilization. Among the questions capable of attack by these techniques are (*a*) what are the sources of the carbons of the glucose molecules comprising glycogen and (*b*) is there a quantitative difference in the sources of the glycogen carbons of livers from diabetic animals compared with normal animals? The intent of this paper is to provide information on these questions.

Topper and Hastings (1) degraded the glycogen formed by liver slices from normal fasted rabbits after incubation with pyruvate-2-C^{14}. From the relative radioactivities of the carbon atoms comprising the glucose, they estimated the proportion of pyruvate molecules (*a*) directly incorporated into glycogen, (*b*) equilibrated with symmetrical dicarboxylic acids before incorporation, and (*c*) converted to $C^{14}O_2$, part of which was eventually incorporated into glycogen. Lorber *et al.* (2) have degraded glucose units of liver glycogen formed from labeled lactate by rats *in vivo*. From the distributions of labeling in the glucose, they calculated the ratio of lactate carbons incorporated directly into glycogen to those that first entered the tricarboxylic acid cycle and then were incorporated.

We have incubated liver slices from normal and alloxan-diabetic rats with pyruvate-2-C^{14} and have degraded both the glycogen formed in the liver and the glucose formed in the medium. From the distribution of activity in the glucose, we have estimated in quantitative terms the extent to which the alternative metabolic pathways were followed by pyruvate in normal liver compared with diabetic liver.

Symmetrical labeling of glucose[1] has been observed in previous degrada-

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[1] Glucose is said to be symmetrically labeled when the activity of carbon 1 = carbon 6, carbon 2 = carbon 5, and carbon 3 = carbon 4.

tions of glycogen formed by liver *in vitro* and *in vivo* from substrates known to enter the glycolytic reactions via glyceraldehyde phosphate (3). However, in the course of our studies the distribution of activity suggested that asymmetry existed in the glucose formed from pyruvate in livers of normal fed rats, the 3 top carbons (C-1,2,3) having less activity than the bottom 3 carbons (C-4,5,6). Asymmetry was not significant in the glucose of glycogen formed in livers of diabetic fed rats. An attempt to understand these observations led us to study the problem of asymmetry in more detail using labeled pyruvate.

EXPERIMENTAL

Male albino rats of the Wistar strain raised in our own colony and weighing between 250 and 350 gm. were used. They were fed standard Purina laboratory chow *ad libitum*. Alloxan diabetes was produced by intravenous injection of alloxan monohydrate after a 24 hour fast. The animals were used 2 to 3 weeks after the injection. Blood sugar values were obtained 2 weeks after administration of alloxan and at death. Fed rats were used only when their blood sugar values were more than 350 mg. per 100 ml. in both determinations.

The incubation medium contained, in millimoles per liter, $K^+ = 110$, $Mg^{++} = 20$, $Ca^{++} = 10$, $HCO_3^- = 40$, and $Cl^- = 90$ or 100, depending on the pyruvate added. Equilibration of the incubation solutions with 5 per cent CO_2-95 per cent O_2 insured a pH in the presence of the liver slices varying between 7.4 and 7.5. The substrates used were (*a*) pyruvate-2-C^{14} (40 mmoles per liter), (*b*) pyruvate-2-C^{14} plus glycerol (each 30 mmoles per liter), and (*c*) pyruvate-2-C^{14} (40 mmoles per liter) plus glucose (20 mmoles per liter). The labeled pyruvate was prepared by Dr. Manfred Karnovsky of this laboratory.

The incubation procedure was the same as that previously described (4). The entire liver of a rat was sliced and between 2 to 3 gm. were placed in each of four incubation flasks containing 12.5 ml. of medium per flask. Incubations were carried out at 37° for a period of 90 minutes. 1 to 2 μc. of radioactive pyruvate was present in each flask.

Isolation of Liver Glycogen and Glucose—The glycogen was isolated by a modification of the method of Good, Kramer, and Somogyi (5) usually by three alcoholic precipitations. Lithium bromide was used to facilitate the final precipitation of the glycogen (6). To livers of fasted rats, 100 to 200 mg. of non-radioactive glycogen were added as carrier. The glycogen was hydrolyzed in 3 ml. of 1 N sulfuric acid for 3 hours on a steam bath. The solution was cooled, diluted with 5 ml. of water, and neutralized with a saturated barium hydroxide solution. After centrifuging, the supernatant liquid was passed over an anion and cation exchange

column after the method described by Abraham, Chaikoff, and Hassid (7). Sufficient non-radioactive glucose was added to make the total amount present in the effluent and washings about 500 mg. The solution was evaporated to a thick glassy syrup, and the glucose was crystallized by addition of warm absolute ethanol and 10 to 20 mg. of non-radioactive glucose as seed after the manner described by Putman *et al.* (8). The amount of crystalline glucose recovered from the glycogen hydrolysate was 90 to 95 per cent of theoretical. It was found that by crystallizing the glucose the subsequent degradation steps were readily handled.

Difficulty in obtaining sufficient radioactive glycogen from diabetic rat liver for total degradation made the availability of a method for isolating glucose from the medium necessary. The medium was deproteinized with $ZnSO_4$-$Ba(OH)_2$ and heat. It was extracted twice with equal volumes of chloroform and then passed down Duolite A-4 and Duolite C-3 exchange columns. The glucose in the effluent was then crystallized as described above for glucose from glycogen.

Degradation of Glucose—Several derivatives of glucose were prepared. From these the activity of a single carbon atom or groups of carbon atoms could be determined. The general scheme employed is outlined in Fig. 1.

Isolation of C-1,2,3 and C-4,5 and C-6—An aliquot of the isolated glucose was converted to glucose phenylosazone, and a portion of this was oxidized by periodic acid to 1,2-bis(phenylhydrazone)mesoxalaldehyde (C-1,2,3) and formic acid (C-4,5), as described by Topper and Hastings (1). A solution of mercuric chloride in acetate buffer (9) was used rather than mercuric oxide to oxidize the formic acid to carbon dioxide. The carbon dioxide was isolated as barium carbonate.

Another portion of the glucosazone was oxidized by periodic acid. The 1,2-bis(phenylhydrazone)mesoxalaldehyde was removed by centrifugation and the supernatant liquid was extracted with chloroform until colorless. The excess periodic acid was destroyed by addition of sodium arsenite and hydrochloric acid. Addition of sodium acetate and dimedon reagent yielded the dimedon of formaldehyde (C-6).

Isolation of C-1 and C-2,3,4 and C-5—Another aliquot of glucose was converted to gluconitrile and this was decomposed to HCN (C-1) and arabinose (1). The HCN was trapped as silver cyanide and oxidized to carbon dioxide with permanganate (10). The carbon dioxide was trapped as barium carbonate. The arabinose was recrystallized from methanol and converted to arabinose phenylosazone by the method of Haskins *et al.* (11). Because the arabinosazone is unstable, after recrystallization from 30 per cent ethanol, it was immediately oxidized to 1,2-bis(phenylhydrazone)mesoxalaldehyde (C-2,3,4) and formic acid (C-5) by periodic

acid in a method analogous to that employed for the oxidation of glucosazone. The formic acid was oxidized to carbon dioxide which was trapped as barium carbonate.

Isolation of C-3—An aliquot of glucose was converted to methyl α-D-glucopyranoside by a modification of the method of Weygand (12). 1 ml.

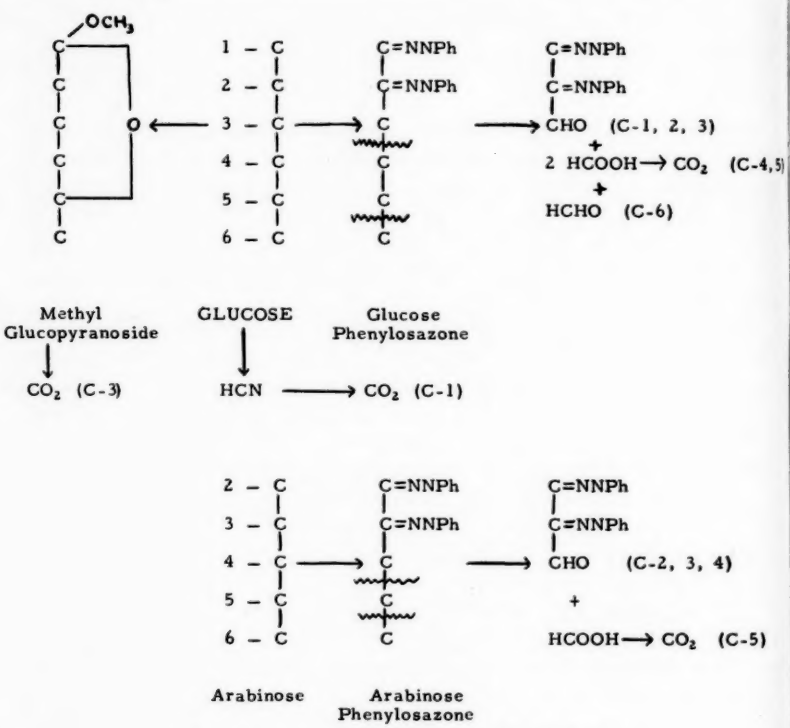

FIG. 1. General scheme of glucose degradation

of 3 per cent hydrochloric acid in anhydrous methanol was added to 100 mg. of glucose. Glucoside formation was carried out by refluxing the mixture for 18 hours. At the end of this period, the solution was cooled and 0.2 gm. of silver carbonate was added. The silver chloride and excess silver carbonate were removed and the supernatant solution was evaporated in a stream of dry air. If crystals did not form on evaporation, a seed of methyl glucoside was added. After crystallization was complete, the methyl glucoside was recrystallized from methanol; yield 31 mg., m.p.

160–163° (reported 166°). The glucoside was oxidized with lead tetraacetate by the method of Abraham (13), yielding C-3 as barium carbonate.

Measurement of Radioactivity—All samples to be assayed were mounted on stainless steel disks which had a plating area of 1.60 sq. cm. Samples of glucosazones, mesoxalaldehydes, and formaldimedons were plated from 30 per cent ethanol and barium carbonate was plated from water. Samples between 3.0 and 8.0 mg. were counted on a proportional flow counter (14). All samples assayed had activities several times background. Counts were corrected with appropriate absorption factors.

Limits of Degradation—Glucosazones, 1,2-bis(phenylhydrazone)mesoxalaldehydes, and barium carbonates were usually plated in duplicate or triplicate. In some cases the degradation was repeated. The values shown in Tables I and II are averages. The activities of duplicates of glucosazones, dimedons, and barium carbonates differed by a maximum of ±5 per cent. 1,2-Bis(phenylhydrazone)mesoxalaldehydes differed by ±10 per cent. Authentic samples[2] of uniformly labeled C^{14}-glucose, glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were subjected to the degradation procedure. The results agreed with those to be expected within the limits just stated, except that they showed that the methyl glucosides oxidized were contaminated by glucose to as much as 4 per cent.

Results

The experimental results on liver slices from twelve rats variously prepared are presented in Table I. The per cent of activity found in the several fractions obtained from degradation of the liver glycogen and the glucose of the medium and the ratio of the activities of C-1,2,3 to C-4,5 plus C-6 (last column) show the following points: (1) No significant difference was found between the distribution of activity in the glucose carbons of the glycogen compared with the distribution of activity of the glucose carbons of the medium in any one experiment. (2) When liver slices from normal fed rats were incubated with pyruvate-2-C^{14} alone (Experiment 1), asymmetry was found (ratio of (C-1,2,3)/-(C-4,5 + C-6) = 0.8). (3) When liver slices from normal fed rats were incubated with pyruvate-2-C^{14} plus non-radioactive glycerol (Experiment 2) or glucose as added substrate (Experiment 3), there was even more marked asymmetry, 0.6 and 0.7, respectively. (4) When liver slices from diabetic fed rats or normal fasted rats were incubated with pyruvate alone, the ratios were unity, indicating that the distribution of radioactivity was symmetrical.

[2] Uniformly labeled C^{14}-glucose was obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Illinois. Glucose-1-C^{14}, -2-C^{14}, and -6-C^{14} were obtained from Dr. H. S. Isbell, National Bureau of Standards, Washington, D. C.

TABLE I

Data on Liver Slices from Rats Prepared under Various Conditions

Experiment No. and conditions	Rat No.	Specific activity of glucosazone	Per cent of activity in fractions							Ratio, $\frac{\text{C-1,2,3}}{\text{C-4,5 + C-6}}$
			C-1,2,3	C-2,3,4	C-4,5	C-1	C-3	C-5	C-6	
		c.p.m. per mmole $\times 10^{-4}$								
1. Pyruvate-2-C^{14}; normal fed	7 G.*	22	43	43	36	15	14	22	21	
	7 M.*	24	44		36				20	
	10 G.	9	42	41	34	15	12	18	17	
	10 M.	37	38		31				18	
Average ± s.e.			42 ± 1	42 ± 1	34 ± 1	15	13	20	19 ± 1	0.8
2. Pyruvate-2-C^{14} + glycerol; normal fed	12 G.	8	36		40					
	12 M.	12	33		40				21	
Average ± s.e.			35		40				21	0.6
3. Pyruvate-2-C^{14} + glucose; normal fed	1 M.	15	40		36				18	
	2 G.	11	40		37	14	13		20	
	2 M.	16	38		39				18	
	3 "	7	36		36	14	12		21	
Average ± s.e.			39 ± 1		37 ± 1	14	13		20 ± 1	0.7
4. Pyruvate-2-C^{14}; diabetic fed	4 G.	13	44		31					
	4 M.	49	46		34	16	13			
	9 G.	17	47		29				18	
	9 M.	76	46		27				18	
	11 G.	8	44		27				16	
	11 M.	18	46	37	31	14	10	21	18	
	15 "	84	48		31		9		18	
Average ± s.e.			46 ± 1	37	30 ± 1	15	11	21	18 ± 1	1.0
5. Pyruvate-2-C^{14}; normal fasted†	13 G.	20	50	41	31	16	12	21	18	
	13 M.	155	48		33				19	
	14 G.	17	50	49	29		12		18	
	14 M.	129	48		32				20	
Average ± s.e.			49 ± 1	45	31 ± 1	16	12	21	19 ± 1	1.0

* G. = glucose from glycogen; M. = glucose from the medium. † Fasted for 24 hours.

According to present concepts of carbohydrate metabolism, the ratio of the specific activities of C-1 to C-6, C-2 to C-5, and C-3 to C-4 should be the same and equal to the ratio of C-1,2,3 to C-4,5,6. Therefore, since the specific activities of C-2 and C-4 were not individually determined, their activities were calculated as follows:

$$\text{C-2} = \frac{(\text{C-5}) \times (\text{C-1,2,3})}{(\text{C-4,5} + \text{C-6})}; \qquad \text{C-4} = \frac{(\text{C-3}) \times (\text{C-4,5} + \text{C-6})}{(\text{C-1,2,3})}$$

Table II presents these derived values for C-2 and C-4 and the experimentally determined figures for C-1, C-3, C-5, and C-6 for the three series in which pyruvate-2-C^{14} was the only substrate. In the lower half of Table II, we have compared the calculated values for C-1,2,3, C-2,3,4, and C-4,5 with those experimentally determined.

TABLE II

Derived and Experimentally Determined Values for C-2,4 and C-1,3,5,6, Respectively, with Pyruvate-2-C^{14} As Substrate

Glucose carbon	Per cent of total C^{14} in glucose carbons					
	Normal fed		Diabetic fed		Normal fasted	
1	15		15		16	
2*	16		21		21	
3	13		11		12	
4†	16		11		12	
5	20		21		21	
6	19		18		19	
	Calculated	Found	Calculated	Found	Calculated	Found
1,2,3	44	42	47	46	49	49
2,3,4	45	42	43	37	45	45
4,5	36	34	32	30	33	31

* C-2 = (C-5 × C-1,2,3)/(C-4,5 + C-6).
† C-4 = (C-3 × C-4,5 + C-6)/C-1,2,3.

From the results, the following statements can be made: (1) There is little difference in the sum of C-1 + C-6 among the three series (33 to 35 per cent). (2) The sum of C-2 + C-5 is greater in the diabetic fed rats and normal fasted rats (42 per cent) than in the normal fed rats (36 per cent). (3) The sum of C-3 + C-4 is greater (29 per cent) in the normal fed rats than in the diabetic fed rats (22 per cent). (4) In general, the distribution in the diabetic fed series is more like that in the normal fasted than in the normal fed series.

DISCUSSION

Fig. 2 shows the presently accepted principal relationships between the substrates, pyruvate and glycerol, and the products, glucose and glycogen. Since liver glycogen and glucose in the medium have a common intermediate, glucose-6-phosphate, it might be expected that glycogen and glucose carbons would have the same relative specific activities. This we have found to be the case.

Pyruvate participates in the glycolytic reactions via glyceraldehyde-3-phosphate and glycerol via dihydroxyacetone phosphate. These two

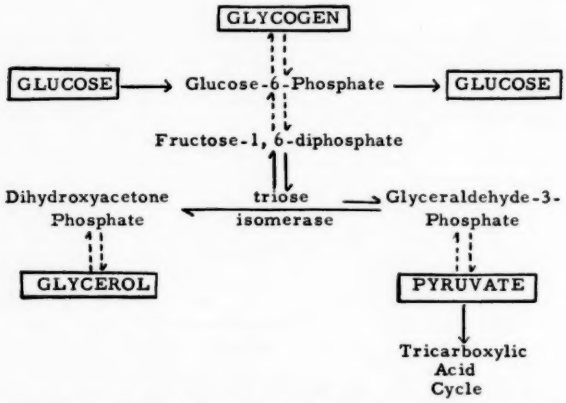

FIG. 2. Principal relationships between the substrates, pyruvate and glycerol, and the products, glucose and glycogen.

trioses are brought to equilibrium by triose isomerase. Condensation of these trioses forms the hexose, fructose-1,6-diphosphate, which is converted to glucose and glycogen. Dihydroxyacetone phosphate contributes C-1,2,3 of the glucose molecule and glyceraldehyde-3-phosphate C-4,5,6. If condensation of labeled glyceraldehyde phosphate (derived from labeled pyruvate) with dihydroxyacetone phosphate (derived from unlabeled sources) occurs before isotopic equilibrium is achieved, the specific activity of C-4,5,6 would be higher than that of C-1,2,3. The larger the non-radioactive 3-carbon pool the greater the asymmetry to be expected. Thus, non-radioactive glycerol would be expected to provide non-radioactive dihydroxyacetone which, if condensed with the radioactive glyceraldehyde, would result in asymmetry. Glucose in the medium or glycogen in the liver would also be expected to provide a non-radioactive 3-carbon pool. Our observations of asymmetry are consistent with such expectations, for as the fasted state was approached the asymmetry decreased.

It is conceivable that Bidder's (15) much discussed observation (16) of asymmetric labeled glucuronic acid formed by liver slices from labeled lactate may have resulted from formation of asymmetric glucose similar to that reported herein.

Observations *in vivo* of Schambye, Wood, and Popják (3) on liver glycogen formed from labeled glycerol by fasted rats indicated that C-1,2,3 of glucose units had a higher specific activity than C-4,5,6. The result obtained in our Experiment 2 *in vitro* with labeled pyruvate and unlabeled glycerol is consonant with the observations *in vivo* of Schambye *et al.* On the other hand, Doerschuk (17) found no evidence of asymmetry in liver glycogen of fed rats to which labeled glycerol had been administered. Furthermore, in preliminary experiments *in vitro* in our laboratory with glycerol-C^{14}, we failed to observe asymmetry in the glucose molecule. This is a problem on which additional experiments *in vitro* would be desirable.

Considerable activity has been found in all the carbons of the glucose molecule formed from pyruvate labeled in only a single position. The amount of activity in C-1 plus C-6 is a measure of the number of pyruvate-2-C^{14} molecules that have entered the dicarboxylic acid shuttle before incorporation into the glucose molecules. The difference in activity between C-2 plus C-5 and C-1 plus C-6 is a measure of the number of pyruvate-2-C^{14} molecules incorporated into the glucose molecules without first entering the tricarboxylic acid cycle. The amount of activity in C-3 plus C-4 is a measure of the amount of pyruvate which was first oxidized to $C^{14}O_2$ and then incorporated into the glucose molecules. We have therefore used the relative activities of the glucose carbons to estimate the relative participation of these reactions of pyruvate in the formation of glucose.

On applying the method of Lorber *et al.* (2) to the normal fasted series (Table II), the distribution of activity of the glucose isolated may be represented as being constituted from two types of isotopic glucose: $\overset{5}{C}—C—\overset{2}{C}—C—C—C$ and $\overset{16}{C}—\overset{16}{C}—\overset{12}{C}—\overset{12}{C}—\overset{19}{C}—\overset{19}{C}—C$. Thus the amount of pyruvate-2-C^{14} that has gone to glycogen directly is about one-thirteenth (7 to 94) of that involved in the reactions of the tricarboxylic acid cycle prior to deposition as glycogen. Lorber *et al.* (2) concluded from their data that, of the labeled lactate carbons administered to rats, less than one-sixth entered the glycogen directly. From the results of Topper and Hastings (1), on incubation of liver slices from fasted rabbits with pyruvate-2-C^{14}, a ratio of 1:7 is obtained. In our diabetic fed series, the ratio is 1:11 and in the normal fed series, 1:50. While these ratios depend upon small differences in C^{14} content, and these differences are not large compared to

the errors present in the methods, apparently much more pyruvate is incorporated directly into glucose in the diabetic fed than in the normal fed. The similarity of distribution of pyruvate activity in the diabetic fed and normal fasted is in agreement with the observations of Renold *et al.* (18) who studied the metabolism of labeled pyruvate and glucose by liver *in vitro* of normal and diabetic rats. It is in accord with their suggestion that in the diabetic liver there is an accelerated conversion of 3-carbon intermediates into glucose.

Using the approach of Topper and Hastings, one can estimate the proportion of pyruvate molecules which are (*a*) phosphorylated directly, (*b*) equilibrated with symmetrical dicarboxylic acids before phosphorylation, and (*c*) converted to $C^{14}O_2$. For the normal fed series this ratio is found to be 1.1:37:62, for the diabetic fed 6:43:51, and for the normal fasted 4.2:42:53. Again, a similarity between the diabetic fed and normal fasted livers and a striking difference in direct phosphorylation between the normal fed and diabetic fed livers are apparent. Thus, in the normal fed rat liver, some 99 of 100 molecules of pyruvate metabolized are involved in the tricarboxylic acid reactions while 1 is phosphorylated directly. In the diabetic fed rat liver, of 100 molecules almost the same number, 94, is involved in the tricarboxylic acid cycle, but 6 are phosphorylated directly. There is no apparent difference in the three series with regard to the proportion of pyruvate molecules entering the shuttle of the tricarboxylic acid cycle. A smaller proportion of pyruvate molecules appears to be oxidized to CO_2 by the liver of diabetic fed animals than by the liver of normal fed animals (51:62). Such a decrease would be in accord with the suggestion of others (19) that there exists a biochemical lesion within the tricarboxylic acid cycle in diabetes.

The possibility exists that some of the glucose-6-phosphate formed in the course of the experiment might have been oxidized to 6-phosphogluconolactone and then metabolized via the direct oxidative pathway (20, 21). We have evidence that this reaction did not occur to a significant degree in our experiments. Dr. James Ashmore, using C-1, C-6, and uniformly labeled glucose, has studied in our laboratory the extent to which glucose is metabolized to carbon dioxide through the oxidative pathway. He finds that the metabolism of glucose by this pathway is less than 10 per cent under our experimental conditions. His results are in agreement with those of Katz, Abraham, Hill, and Chaikoff (22).

SUMMARY

Rat liver slices were incubated with pyruvate-2-C^{14} under a variety of experimental conditions. Crystalline glucose was isolated from the glycogen formed in the liver and the glucose formed in the medium. The

glucose was degraded so that the specific activity of all its carbon atoms could be determined.

1. The distribution of activity in the glucose from the glycogen was the same as that of the glucose from the medium for each incubation.

2. With pyruvate-2-C^{14}, asymmetrical labeled glucose was obtained, the extent of the asymmetry appearing to depend on the availability of non-radioactive trioses.

3. The distribution of activity in the glucose and glycogen formed by liver slices from normal fed, diabetic fed, and normal fasted rats with pyruvate-2-C^{14} as substrate was determined. The data indicate that a relatively larger proportion of pyruvate metabolized is directed to glucose by the liver of diabetic and fasting animals than by that of normal fed animals.

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XUM

STUDIES OF THE UROPORPHYRINS

IV. REEXAMINATION OF A CRUCIAL WALDENSTRÖM PORPHYRIN*

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The Waldenström porphyrin of urine from cases of acute porphyria was initially believed to be uroporphyrin III (1–3). Grinstein and coworkers (4) and Watson, Schwartz, and Hawkinson (5) reported that it was an association or complex of uroporphyrin I with a smaller fraction of a type III isomer containing seven COOH groups. The preponderance of type I isomer in the complex was subsequently confirmed by others (6–8), but very recently Rimington and coworkers, while acknowledging that the Waldenström porphyrin is an association of type I and III isomers, have described evidence indicating that uroporphyrin III comprises approximately 75 per cent and uroporphyrin I 25 per cent of the complex. This conclusion was based on comparisons with mixtures of uroporphyrin I from congenital (erythropoietic) porphyria urine and uroporphyrin III from turacin (9). Mixed melting point curves (10), x-ray crystal powder patterns (11), infra-red spectra, and the Falk-Benson paper chromatographic method with two solvent pairs (12) were employed. It was assumed that preliminary MgO chromatography (13) was adequate to insure the sole presence of a (uro) porphyrin with eight COOH groups. It may be noted, however, that Rimington[1] and coworkers, either with this or other methods, have observed a 7-COOH porphyrin only in insignificant amounts. Grinstein *et al.* (4) described a 7-COOH or "208" porphyrin (ester) as a significant component of the Waldenström porphyrin type A complex; *i.e.*, that resolved by $CaCO_3$ chromatography into a major fraction of uroporphyrin I, and a minor fraction, the "208", type III isomer. The type B complex, although found to include similar proportions of type I and III isomers on decarboxylation, behaved as an entity on $CaCO_3$ (5). In 1950, Gray (7) isolated a uroporphyrin of the latter type from the urine of a case of acute porphyria (Hannah S.) and reported that it was preponderantly uroporphyrin I. Rimington[1] has recently studied Gray's porphyrin by the methods

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[1] Personal communications.

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mentioned above and concludes that it is mainly a type III uroporphyrin. Rimington's study included decarboxylation, the results of which were described[1] as follows: "The copro- fraction was subjected to one crystallization from $CHCl_3$-CH_3OH with concentrated solutions. Crystals were deposited with melting point rather indefinite from 220–234° and an *intensely* colored mother liquor remained. Chromatography of the crystals and mother liquor separately by the Chu method showed mainly coproporphyrin I + some III in the crystals and mainly coproporphyrin III + some I in the mother liquors." On this basis it was concluded that the coproporphyrin obtained was "clearly mainly coproporphyrin III with some coproporphyrin I also present." No quantitative data are available.

EXPERIMENTAL

Professor Rimington kindly provided us with 11 mg. of the Gray uroporphyrin ester, and, since it is obviously of crucial importance with respect to the differing results just referred to, a careful reexamination has been made as to the amounts of coproporphyrins I and III provided upon decarboxylation. It was first ascertained that, in agreement with Rimington's findings, the Gray porphyrin ester behaved entirely as a uroporphyrin by MgO chromatography as performed according to Nicholas (13). By the Falk-Benson paper chromatographic method (12) it behaved as a 40:60 mixture of uroporphyrin I and uroporphyrin III[2] (turacin). Rimington[1] reported the melting point to be 255–258°; we noted 244–250°, with some charring above 224°. This was unchanged after MgO chromatography, but a definite change was noted after purification on $CaCO_3$. The material behaved as an entity, or "type B Waldenström complex" (5), moving down on the column as a single zone. A very small fraction, presumably unesterified, remained on top and was not identified. The Waldenström ester was eluted in $CHCl_3$; α absorption 626.1 mμ (maximum), m.p. (from $CHCl_3$ methyl alcohol) 260–262°; after four recrystallizations, 261–263°.

X-ray crystal powder patterns were studied and compared with those of uroporphyrin I and III[3] (turacin) methyl esters. The interplanar spacings are recorded in Table I. It is believed significant that the patterns from MgO alone, and from MgO- and $CaCO_3$-purified material, are distinct. It is also evident that neither pattern is identical with that of uroporphyrin I or III.

Decarboxylation was carried out on material both as it was received and

[2] Provided through the courtesy of Professor Rimington. As will be described separately, we have not thus far been able to isolate a homogeneous or unequivocal uroporphyrin III from any source, including various samples of turacin available to us.

[3] Provided through the courtesy of Professor Rimington (see foot-note 2).

after $CaCO_3$ chromatography (Runs *a* and *b*, as discussed in the following). The improved procedure of Edmondson and Schwartz (14) has been used, as, with amounts up to 5 mg., this usually permits a much more efficient conversion to coproporphyrin.

In Run *a* 4.3 mg. were decarboxylated. The tube broke in the oven, owing probably to too rapid heating. 4.2 mg. were recovered, resealed, and reheated in 0.3 N HCl. After cooling, the reaction was adjusted to

TABLE I

x-Ray Crystal Powder Patterns of Gray Uroporphyrin Methyl Ester

Interplanar spacings in angstrom units.

1st sample as received	After MgO chromatography	After MgO and subsequent $CaCO_3$ chromatography	Uroporphyrin I	Uroporphyrin III*
1.77 W.	1.77 W.	2.05 M.	1.81 W.	1.76 W.
2.06 "	2.05 M.	3.34 W.	1.93 "	2.04 M.
2.75 "	3.17 W.	3.65 "	2.11 "	3.11 "
3.19 "	3.66 M.	3.90 M.	2.22 "	3.66 "
3.43 "	4.14 "	4.14 S.	2.72 "	3.88 W.
3.69 "	4.38 W.	4.42 M.	3.02 "	4.06 S.
4.14 S.	4.70 M.	4.67 "	3.17 M.	4.21 "
4.25 M.	5.08 "	5.20 "	3.44 "	4.65 "
4.47 "	8.16 "	8.32 W.	3.65 "	5.17 "
4.70 S.			3.85 "	5.81 W.
5.20 "			4.10 S.	8.16 "
5.73 W.			4.47 M.	
8.24 "			4.80 "	
			5.26 "	
			5.81 W.	
			8.16 "	

W. = weak; S. = strong; M. = medium.

* See foot-note 2.

pH 4.0 by addition of NaOH and $NaOOCCH_3$, and the porphyrin was removed by ether extraction. All but 31.5 γ was ether-soluble. This small fraction was discarded. The porphyrin in the ether phase was extracted by 1.5 N HCl, which was mixed with 10 volumes of a mixture of CH_3OH and H_2SO_4 (19:1), and allowed to stand overnight. The porphyrin ester was then extracted with $CHCl_3$ and this solution worked up in the usual way (4). After concentration to dryness under reduced pressure, the residue was redissolved in C_6H_6, and a few drops of petroleum ether were added. $CaCO_3$ chromatography was then carried out in the usual way (4, 5). A small brown, non-fluorescing zone remained on top during elution with $CHCl_3$-C_6H_6 (1:9). This was not further identified. A broad

single red fluorescing zone moved downward on the column. When about half way, the column was allowed to dry and the red fluorescing zone was cut out (4). The porphyrin ester was eluted from the $CaCO_3$ with $CHCl_3$. After concentration and addition of hot methyl alcohol, the porphyrin ester crystallized readily in the fluffy, hair-like needles characteristic of coproporphyrin I methyl ester. The yield of the first crystallizations, determined fluorometrically as coproporphyrin, was 2.4 mg.; m.p. of crystals, 220–240°. After two recrystallizations the melting point was 242–246°. The crystals were now washed with cold absolute ether, in which coproporphyrin III methyl ester is much more soluble than type I. The ether was almost colorless and contained but 100 γ of coproporphyrin, the great majority being insoluble. After a third recrystallization from $CHCl_3$-CH_3OH the melting point was essentially unchanged at 244–246°, and as the molten material cooled it became completely crystalline again at 233°. As Jope and O'Brien (15) have shown, these melting and solidification points indicate at least 95 per cent coproporphyrin I. The melting point of pure coproporphyrin I methyl ester is generally given as 250°, while coproporphyrin III ester has a dimorphic melting point: 148°; remelted, 172°. The combined mother liquors contained 0.39 mg. of coproporphyrin. The final yield was 1.68 mg., representing 50 per cent of the theory (4.2 mg. of uroporphyrin equivalent to 3.3 mg. of coproporphyrin). This yield, however, represented approximately 68 per cent of the total coproporphyrin obtained from the $CaCO_3$ column. By the Chu-Green paper chromatographic method (16) the 0.49 mg. in the mother liquors and ether wash contained more type III than type I, but, as seen in Fig. 1, the behavior was atypical for coproporphyrin. By the fluorescence quenching technique (16) the amounts of the two isomers were about equal. The combined porphyrin from these fractions crystallized slowly from methyl alcohol in small rosettes (coproporphyrin III habitus). After two recrystallizations the melting point was over the range 160–205°. On cooling the melt, the material did not crystallize sharply. It was evident in Run *a* that some loss had occurred; thus comparison with the results in Run *b* are believed significant, since in this run no difficulty was encountered, such as the accident at the outset in Run *a*. However, in Run *b* there was also the difference that the uroporphyrin ester was subjected to $CaCO_3$ chromatography prior to decarboxylation. The amount decarboxylated was 5 mg. The procedure and subsequent treatment were as given above for Run *a*. Only a trace of porphyrin was insoluble in ether after decarboxylation. The total porphyrin ester behaved as a single zone on $CaCO_3$, a trace of unidentified material remaining on top of the column. The fluffy hair-like crystals from $CHCl_3$-CH_3OH melted at 247–250°. The mother liquor was only faintly colored. The maximum of the α absorption band of the crystals

dissolved in $CHCl_3$ was 622.1 mμ, identical with that of coproporphyrin ester. After three recrystallizations the melting point was constant at 251–252°. On cooling the melt, the material became crystalline from 235–230°. The yield was approximately 3.4 mg. The combined mother liquors

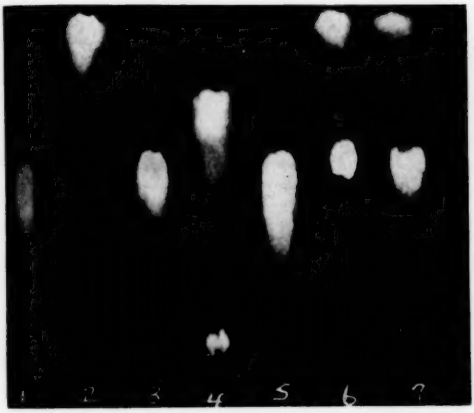

FIG. 1

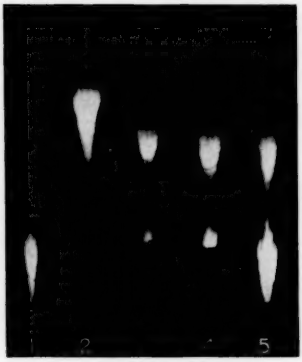

FIG. 2

FIG. 1. Paper chromatogram of decarboxylation products from Runs *a* and *b*, by Chu-Green method (16). Red fluorescence photographed under ultraviolet light (General Electric flood light No. E.H4 with Corning Corex A filter, secondary yellow filter No. 3484 at camera). *1*, coproporphyrin I; *2*, coproporphyrin III; *3*, Run *a*, recrystallized four times; *4*, Run *a*, upper brown zone; *5*, Run *b*, recrystallized four times; *6*, mother liquor from Run *b* (68 per cent coproporphyrin III by fluorescence quenching technique); *7*, 50 per cent coproporphyrin I, 50 per cent coproporphyrin III.

FIG. 2. Paper chromatogram by Falk-Benson method of Gray uroporphyrin employed in Runs *c* and *d* compared with reference porphyrins. *1*, uroporphyrin I; *2*, "208" porphyrin with a trace of 6-COOH porphyrin; *3*, Gray porphyrin after MgO, as used in Run *c*; *4*, Gray porphyrin after MgO and $CaCO_3$, as used in Run *d*; *5*, artificial mixture of equal parts of uroporphyrins I and III (turacin, Rimington's). All of the above porphyrins were methyl esters.

contained but 0.12 mg. of porphyrin. Chu-Green chromatography of this fraction showed type III > I; fluorescence quenching 70 per cent type III.

Further runs (*c* and *d*) were made on an additional 6 mg. sample of the same uroporphyrin ester, which was kindly supplied by Professor Gray. The melting point of the material as received was 248–255°. By Falk-Benson chromatography it behaved as a mixture of uroporphyrins I and III[3] (30:70) (Fig. 2). After MgO chromatography, 250–257°; after MgO and subsequent $CaCO_3$ chromatography, 258–262°. Run *c* was made on

2.5 mg. of the former (MgO only) and Run *d* on 2.34 mg. of the latter (MgO and $CaCO_3$), each being decarboxylated individually. Subsequent treatment of each was the same as above for Runs *a* and *b*. The product in each instance was almost wholly ether-soluble, giving the coproporphyrin spectrum. The methyl ester was crystallized in each instance, as seen in Table II.

The final yield of coproporphyrin from each of the above runs was studied by infra-red spectrometry.[4] In each instance identity with copropor-

TABLE II

Coproporphyrins from Runs c and d (Material Obtained from Professor Gray)

	Run *c*, MgO only	Run *d*, MgO + $CaCO_3$
Uroporphyrin decarboxylated, *mg.*	2.5	2.34
M.p. after 1st crystallization, *°C.*	220–239 (mother liquor red-brown)	235–242 (mother liquor light red)
Times recrystallized	8	5
Final m.p., *°C.*	241–244	244–247
Yield, *mg.*	1.12	1.15
Mg. in combined mother liquor	0.3	0.10
Yield of crystals, coproporphyrin I, *% of theory**	60	65.5
Proportion of isomers types I, III		
Chu-Green method (16)		
Crystallization	I	I
Mother liquor	III > I	III > I
Fluorescence quenching (Schwartz *et al.* (17)), % III		
Crystallization	17	5
Mother liquor	75+	75+

* Uroporphyrin to coproporphyrin.

phyrin I methyl ester was observed, the spectra being easily distinguished from that of coproporphyrin III. Interplanar spacings for the x-ray crystal powder patterns from Runs *c* and *d* are given in Table III.

From the above data it appears that a slightly better yield of purer material resulted after $CaCO_3$ chromatography. The findings in Runs *a* and *b* also indicate that the accident during decarboxylation was also a factor in reducing the yield and producing a complex mixture. This sim-

[4] Studied through the courtesy of Professor Bryce Crawford and Mr. R. C. Golike, Department of Physical Chemistry, University of Minnesota. The curves were obtained with Nujol mull preparations and a Perkin-Elmer infra-red spectrometer, model 12C, modified for double pass.

ply emphasizes that differences in technique may give rise to some confusion, although this was insufficient to affect the main conclusion that type I isomer was preponderant.

TABLE III

*x-Ray Crystal Powder Patterns of Coproporphyrin Obtained by Decarboxylation of Gray Uroporphyrin, Compared with Four Standard Coproporphyrin Isomers**

All porphyrins were studied as the methyl esters.

Coproporphyrin I	Coproporphyrin II	Coproporphyrin III	Coproporphyrin IV	Coproporphyrin from Gray uroporphyrin	
				Run *c*	Run *d*
1.70 W.	2.06 M.	3.06 S.	2.77 W.	2.05 W.	1.77 W.
2.07 "	2.84 W.	3.29 "	3.19 S.	2.31 "	2.04 "
2.32 "	3.29 M.	3.52 W.	3.77 "	2.77 "	2.32 "
2.39 "	3.42 S.	3.67 S.	4.14 "	2.93 "	2.75 "
2.77 M. W.	3.56 W.	3.84 "	4.36 "	3.02 M.	2.92 "
2.91 W.	3.75 S.	4.03 W.	4.60 W.	3.21 W.	3.02 M.
3.00 S.	3.95 "	4.19 "	5.26 "	3.49 "	3.21 W.
3.19 M.	4.17 "	4.44 S.	5.81 "	3.77 S.	3.35 "
3.34 W.	4.38 M.	4.86 "	7.00 M.	3.90 W.	3.52 "
3.52 "	4.60 "	5.29 M.	8.32 "	4.10 S.	3.75 S.
3.76 S.	4.80 "	5.89 S.	11.30 W.	4.25 W.	3.88 W.
3.89 W.	5.45 S.	6.59 W.	13.77 "	4.70 S.	4.08 S.
4.12 S.	6.04 W.	7.23 S.		4.96 W.	4.25 W.
4.68 "	9.18 S.	8.17 W.		5.20 "	4.70 S.
4.97 W.	11.30 W.	9.58 S.		5.52 M.	4.86 W.
5.45 M.				5.88 W.	5.14 "
5.89 M. W.				6.49 "	5.45 M. W.
6.44 "				8.64 "	5.88 W.
7.17 W.				9.48 "	6.39 "
8.32 "					7.60 "
9.38 "					8.64 "
					9.38 "

* Type I isolated from feces of erythropoietic (congenital) porphyria; Type III from diphtheria bacilli; Types II and IV provided through the courtesy of Professor A. Treibs, Organisch-chemisch Institut der technischen Hochschule, Munich, Germany.

DISCUSSION

So far as can be determined, Rimington's conclusion that the coproporphyrin obtained from Gray's uroporphyrin is mainly type III (see above) is based on the relatively large proportion of porphyrin remaining in the (methanol) mother liquor, together with the result of the Chu-Green method (16) applied to this porphyrin. The latter technique, while of con-

siderable value, has distinct limitations, at least in our experience: (1) with amounts less than 0.4 γ, there is an increasing error in favor of type III; the opposite is true as the amount is increased above 0.5 γ; (2) if the coproporphyrin ester has not been crystallized quite recently, the result may differ significantly from that obtained with freshly purified material. This indicates that minor alterations or impurities may affect the method significantly. With respect to the relative amount of coproporphyrin I and III esters remaining in the mother liquor, it may be noted that in exceptional instances, especially on the first crystallization, the amount of coproporphyrin I remaining in solution is surprisingly large. While the color intensity of the first mother liquor noted in Runs *a*, *b*, and *d* was not as great as that indicated by Rimington, it is seen that considerably more porphyrin remained in the mother liquor in Run *a* than in Run *b*, also in Run *c* than in Run *d*. In fact, the data actually suggest that a larger amount of coproporphyrin III was formed in Run *a* than in Run *b*. While the spectroscopic data and behavior on MgO and $CaCO_3$ failed to indicate the presence of any other than coproporphyrin, the behavior by Chu-Green chromatography indicated a distinct difference (Fig. 1). It is believed quite unlikely that any actual difference in isomer ratio was induced, but it does appear that a more complex mixture of porphyrins, or of porphyrins and impurities, occurred in Run *a* compared with Run *b*.

We have repeatedly noted significant differences between $CaCO_3$ and MgO in respect to the behavior of the Waldenström porphyrin associations. Thus the type A Waldenström porphyrin which is resolved on $CaCO_3$ into uroporphyrin I and the "208" porphyrin regularly behaves as an entity on MgO, with the Nicholas method. It is also of considerable importance that the type A porphyrin often runs quite similarly to uroporphyrin III by Falk-Benson paper chromatography, despite the fact that uroporphyrin I is its major component when resolved on $CaCO_3$.

Cookson and Rimington (18) state that the uroporphyrin obtained by heating crystalline porphobilinogen (19) *in vitro* is mainly uroporphyrin III. As described in detail separately, the methyl ester of the coproporphyrin (or coproporphyrins) which we have obtained on decarboxylation of this uroporphyrin behaves quite differently from the corresponding mixtures discussed in the foregoing and regularly obtained by decarboxylation of the Waldenström porphyrin from acute porphyria urine. The coproporphyrin ester mixture from the porphobilinogen uroporphyrin is characterized by an unusual degree of solubility in methyl alcohol, the mother liquor being darkly colored. This seriously interferes with recrystallization of small amounts from $CHCl_3$-CH_3OH, although the ester crystallizes well from benzene-petroleum ether (b.p. 30–60°). The melting point of the crystals obtained in either way is not sharp, at first from 160–220°; with

repeated recrystallization, from 160–190°. We have not been able to prepare a pure coproporphyrin III from this mixture. Even more important however, has been the striking paucity of coproporphyrin I, with its characteristic relative insolubility in methyl alcohol and high melting point, as noted above and previously (4, 5). This indicates that the uroporphyrin formed by heating crystalline porphobilinogen, either in the solid state or in acid aqueous solutions, differs significantly from the Waldenström porphyrin in acute porphyria urine or formed during the process of isolation. It should be noted that the term Waldenström porphyrin was first employed by us (4, 5) to designate the porphyrin isolated by Waldenström's and other methods from *unheated* human porphyria urine. According to Gray (7), the Waldenström ester, which is the subject of the present study, was obtained from urine kept at refrigerator temperature. Studies which are still in progress indicate that heating the urine according to Rimington introduces significant differences in the direction of formation of a greater proportion of isomers other than type I. This work will be reported in a separate communication. It may be noted, however, that with the presence of type II and IV isomers in a Waldenström complex formed by heating porphobilinogen (20) it would be anticipated that the resultant crystalline ester would have physical characteristics differing from a simple mixture of uroporphyrins I and III, such as was used by Rimington *et al.* (10–12) as a reference compound in studying the mixed melting point, x-ray crystal powder pattern, and Falk-Benson paper chromatographic behavior of Waldenström esters from acute porphyria urine.

SUMMARY

1. A reexamination has been made of a Waldenström porphyrin isolated originally by C. H. Gray from the urine of a case of acute porphyria. Although Gray reported that this porphyrin was chiefly type I isomer, it was subsequently stated by Rimington to be mainly type III.

2. Calcium carbonate chromatography of this porphyrin ester was found to elevate the melting point and alter the x-ray crystal powder pattern as compared with material obtained after magnesium oxide chromatography alone.

3. By Falk-Benson chromatography the porphyrin behaved as though a major portion of the complex were type III. Semiquantitative decarboxylation revealed, however, that the resulting coproporphyrin was mainly type I, as identified by ester melting point, Chu-Green chromatography, fluorescence quenching behavior, infra-red spectrum, and x-ray crystal powder patterns. From this it is concluded that the original uroporphyrin complex was preponderantly type I.

XUM

The technical assistance of Miss Ruth Cardinal is gratefully acknowledged.

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STUDIES OF THE UROPORPHYRINS

V. THE PREPARATION AND PROPERTIES OF AN ARTIFICIAL WALDENSTRÖM PORPHYRIN*

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As discussed in the preceding paper, the Waldenström uroporphyrin complex obtained from acute porphyria urine (1–3) often behaves mainly as a type III isomer when subjected to the Falk-Benson chromatographic procedure (4), even though decarboxylation, on a semiquantitative basis, reveals that the molecular complex contains a preponderance of type I. The Falk-Benson method was stated to separate the methyl esters of uroporphyrins I and III, the former as isolated from erythropoietic (congenital) porphyria urine, the latter from certain samples of turacin. This method is an adaptation of the Chu-Green procedure (5), which has been used to separate coproporphyrin I and III methyl esters on paper. The Waldenström complex behaves mainly as uroporphyrin III (from turacin) by the Falk-Benson method, regardless of whether it is type A or B by $CaCO_3$ chromatography; in other words, whether it is resolved into a major fraction of uroporphyrin I, and a minor fraction of the so called "208" porphyrin (see below), or whether it behaves as an entity (2, 3). The Waldenström complex isolated by Gray (6), as studied in the preceding paper, belonged in the latter category. Waldenström porphyrins of both types have now been isolated by a variety of methods from a number of cases of porphyria, both intermittent acute and "cutanea tarda," to permit reexamination by newer methods, including the Falk-Benson technique. The results of this study will be described in the next paper of this series. It will suffice to note at present that these studies confirm the observation mentioned above; *i.e.*, that the uroporphyrin complex isolated by Waldenström's method (1) and certain other procedures not employing heat is often indistinguishable on paper chromatography from uroporphyrin III, even though subsequent decarboxylation and examination of the coproporphyrins obtained clearly revealed that the complex was preponderantly type I isomer. This suggested that it would be of interest to mix uropor-

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phyrin I and the "208" type III (heptacarboxyl) porphyrin in proportions similar to those which have been observed in the type A Waldenström porphyrin and to note the behavior of this mixture under various circumstances. Accordingly the following experiments were carried out.

EXPERIMENTAL

The uroporphyrin I employed had been isolated from the urine of a typical case of erythropoietic (congenital) porphyria. This was Case 72 of our total porphyria cases and has been described elsewhere in detail (7, 8). The methyl ester was recrystallized repeatedly from chloroform-methyl alcohol. The melting point was 288–292°. This behaved as uroporphyrin I by Falk-Benson chromatography. On decarboxylation nearly all of the coproporphyrin formed was type I isomer; the fluorescence quenching technique indicated the presence of traces of type III, while the Chu-Green method indicated all type I.

The "208" porphyrin was obtained from a pool of type A Waldenström porphyrin isolated as follows from four cases of intermittent acute porphyria (Cases 42, 138, 141, and 158 (8)); 4 day urine collections were obtained from each, alkalized with Na_2CO_3 from the outset of collection. The individual collections were adjusted to pH 4.0 with acetic acid, and the preformed porphyrin was adsorbed by shaking with several batches of talc. The residual urine contained the porphobilinogen, which was isolated by Westall's method (9). The talc from all of the collections was pooled, and the porphyrin was eluted with CH_3OH-H_2SO_4 (19:1). After standing overnight, the eluate was mixed with a small amount of $CHCl_3$ and a large volume of water in a separatory funnel. The $CHCl_3$ was washed with water, ammonia, and NaCl solution in the usual way (2), the filtered $CHCl_3$ solution was concentrated to dryness under reduced pressure, and the total porphyrin dissolved in benzene. After adding a few drops of petroleum ether, the solution was run through a $CaCO_3$ column as described previously (2, 3). It has been known for years that many samples of $CaCO_3$ are unsuitable for this purpose or even for separation of uro-, copro-, and protoporphyrins. A brief discussion is warranted as to the selection of the $CaCO_3$. It has recently been found that the iodine number, as applied to MgO for other chromatographic purposes, is useful in ascertaining the value of any given sample of $CaCO_3$ for porphyrin work. The iodine number is defined as millimoles of I_2 adsorbed by 1 gm. of the material being studied, $\times$ 100, after being shaken for 5 minutes with 20 ml. of CCl_4 containing 2 mg. of I_2. Of six samples of $CaCO_3$ of various origins, the range of iodine number was from 130 to 208. The samples ranging from 170 to 177 proved very satisfactory in separating uroporphyrin I, the "208" porphyrin, copro- and protoporphyrin; three, with iodine numbers of 138, 140, and 149, were

not retentive enough and two of 191 and 208 were too retentive. Merck U. S. P. $CaCO_3$, No. 52552, dried at 37° in thin layers, had an iodine number of 177 and proved very satisfactory. The spread of values with MgO is greater and thus of even greater value.[1] Three samples have been studied: Merck Nos. 51R6180, 51R6181, and 51R6182. The iodine numbers reported by Merck and Company[2] were 114, 29, and 54, respectively. We found 110, 25, and 50; thus it is evident that the method is reproducible within reasonable limits. Of these three only the second (iodine number 25) was satisfactory, and this permitted excellent separation of uro-, copro-, and protoporphyrin according to Nicholas' method (10). It may be emphasized, however, that it has not been possible to resolve the type A Waldenström porphyrin into uroporphyrin I and the "208" porphyrin with MgO, as is regularly achieved with a suitable $CaCO_3$.

With the above pool of Waldenström type A porphyrin ester, the first $CaCO_3$ column achieved separation of a broad zone of uroporphyrin methyl ester (α absorption band at 626.1 mμ) and coproporphyrin (622.2 mμ). On rechromatographing the uroporphyrin fraction, a relatively narrow zone separated below the main uroporphyrin, in the manner previously noted (2). The α absorption of this zone, diluted in $CHCl_3$, was 625.6 mμ. After three recrystallizations the melting point was from 205–209° and the yield approximately 4 mg. The upper uroporphyrin zone eluted in $CHCl_3$ had an α absorption band at 626.0 mμ, and upon repeated recrystallization the melting point of the methyl ester was 278–282°. The yield was approximately 14 mg.

Experiment 1—2.1 mg. of the above ester melting at 205–209° (*i.e.*, the "208" porphyrin ester) were dissolved in a small amount of $CHCl_3$, and this was mixed with 8.4 mg. of uroporphyrin I methyl ester in $CHCl_3$. The volume was 6 ml. The amount of 0.3 ml. was removed for various studies, including chromatography on $CaCO_3$ (2, 3), MgO (10), and the Falk-Benson paper chromatographic method (4). The remainder was concentrated to dryness under reduced pressure with but slight heating,

[1] A much greater spread is obtained for $CaCO_3$ by the use of coproporphyrin I or mesoporphyrin 9 rather than I_2, the methyl ester of either porphyrin being used in the ratio of 1:1000 with $CaCO_3$. It is convenient to use 0.1 mg. of ester in 15 ml. of benzene and to shake the solution with 100 mg. of $CaCO_3$. The porphyrin number is the per cent removed by the $CaCO_3$. A sample (Merck, U. S. P. precipitate), which resolved porphyrin mixtures very well and separated the "208" porphyrin, had a coproporphyrin number of 34; for another that was much too retentive the number was 88 and a third, not sufficiently retentive, had a porphyrin number of 10. The same three $CaCO_3$ samples had mesoporphyrin numbers of 61, 80, and 34, respectively.

[2] Information as to the I_2 number and also various samples of $CaCO_3$ and MgO were kindly supplied by Dr. Robert Peck, Merck and Company, Inc., Rahway, New Jersey.

and the total porphyrin ester was saponified by dissolving it in 7.5 N HCl and allowing the solution to stand overnight. This solution was then neutralized partly with NaOH and $NaOOCCH_3$, finally with Na_2CO_3 solution, the reaction at the end being adjusted to pH 8.0. This solution, measuring 50 ml., was added to 150 ml. of freshly passed normal urine, pH 7.0. The final pH was slightly less than 8.0. The mixture was refrigerated at 4° for 2 days and then worked up as follows: The pH was adjusted with HCl to 3.2 ± 0.2 (gray-blue to Congo paper). Three extractions with ethyl acetate were then made, partial emulsions being separated by small amounts of ethyl alcohol. About 90 per cent of the total porphyrin was extracted. Most of the porphyrin was removed from the ethyl acetate by repeated shaking with 1 per cent sodium acetate solution; a very small fraction was then extracted with 1.5 N HCl. These solutions were combined. The ethyl acetate solution was irradiated for 5 minutes in a Pyrex flask with a mercury arc lamp. No further porphyrin appeared, showing that no reduction of the porphyrin had taken place during the period of exposure to the urine. The aqueous extract was adjusted to pH 4.0 ± 0.2 with acetic acid mixed with 0.5 volume of acetone, and run through a column of Al_2O_3 (Merck's for chromatography), which was first washed with 50 per cent acetone. The total porphyrin was eluted and esterified with methyl alcohol-H_2SO_4 (19:1), and the methyl ester then taken into $CHCl_3$ as described above. After washing with water, ammonia, and NaCl solution, the $CHCl_3$ solution was then concentrated under reduced pressure and subjected to MgO chromatography (10). All of the porphyrin ester was eluted by 0.5 per cent methyl alcohol in $CHCl_3$; in other words, as a uroporphyrin. The solution contained 6.15 mg. The ester behaved as an entity or "type B Waldenström ester" (3) on a $CaCO_3$ column. The crystals from $CHCl_3$-CH_3OH melted at 258–262°, essentially unchanged on recrystallization. The mother liquor was almost colorless and contained but 42 γ of uroporphyrin. The α absorption band of the crystals in $CHCl_3$ was located at 626.2 mμ (maximum).

Falk-Benson chromatography revealed that with the amounts found to be satisfactory for uroporphyrins I and III this artificial Waldenström complex behaved mainly as type III, despite its known composition of 80 per cent type I (Fig. 1). It is seen that with increasing concentrations on paper a greater proportion behaved as type I, the majority, however, still running as type III. It was believed highly significant that there was no spot corresponding with that of the "208" porphyrin ester when run alone or in simple mixture with uroporphyrin I ester unexposed to urine. This is regarded as further evidence of a molecular association.

5.0 mg. were subjected to decarboxylation by the improved technique of Edmondson and Schwartz (11). All of the porphyrin was now ether-

soluble and after esterification exhibited but one zone on $CaCO_3$. The chromatographed product crystallized readily from $CHCl_3$-CH_3OH. Quantitative and other data for crystallization and a number of recrystallizations are presented in Table I.

Experiment 2—Uroporphyrin I ester of the same source and characteristics as in Experiment 1 was mixed with a "208" porphyrin ester isolated in the same way from a case of "mixed" hepatic porphyria. This individual, Case 86 in our series (8), a male aged 48 years, had previously shown photo-

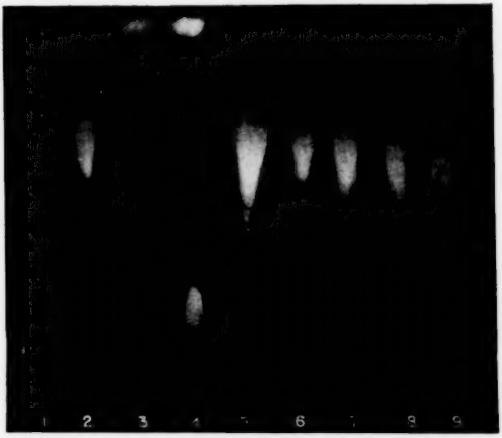

FIG. 1. The Falk-Benson method applied to the artificial Waldenström porphyrin methyl ester as compared with the esters of other porphyrins. *1*, uroporphyrin I 0.5 γ; *2*, uroporphyrin III 0.5 γ; *3*, "208" porphyrin 0.5 γ; *4*, uroporphyrin I + "208" (4:1) 0.5 γ; *5*, uroporphyrin I + uroporphyrin III (1:3) 0.5 γ; *6*, artificial Waldenström porphyrin (Experiment 1) 0.1 γ; *7*, same, 0.3 γ; *8*, same, 0.5 γ; *9*, same, 1.0 γ.

sensitivity and cutaneous lesions, but at the time of isolation of the "208" porphyrin ester had only abdominal and leg pain of intermittent character, despite the same degree of light exposure as before. Reasons for this difference in relation to hepatic porphyrin and precursor content have been discussed elsewhere (8). A pool of Waldenström type A porphyrin ester of about 30 mg., obtained from many 24 hour urine samples from this patient, served as the source of "208" porphyrin ester; m.p. 206–208°. The melting point of the Waldenström ester itself was constant at 258–260° after several recrystallizations. A number of $CaCO_3$ columns were necessary to separate the entire amount into its constituent uroporphyrin I and "208" porphyrin esters. 6 mg. of the latter were obtained as the methyl ester. Of this, 2.06 mg. were mixed with 8.0 mg. of uroporphyrin I ester as in Experiment

1. After saponification and adjustment of pH to 9.0 the 25 ml. solution of free porphyrin was mixed with 75 ml. of normal urine (from a different subject than in Experiment 1). The final pH was approximately 8.0. The mixture was refrigerated at 4° overnight and then acidified with HCl and extracted with ethyl acetate as before. Although the major part of the porphyrin passed into the organic solvent, a considerable amount, much more than in Experiment 1, remained in the urine. The ethyl acetate fraction, worked up as in Experiment 1, yielded 3.48 mg. of crude, crystalline ester. The material remaining in the urine was recovered by adsorbing it, after adjustment of the pH to 4.0, on talc, and then eluting it with

TABLE I

Data Relating to Crystallization of Coproporphyrin Esters Obtained by Decarboxylation of Artificial Waldenström Porphyrin Obtained in Experiment 1

Times crystallized	M.p.	Mother liquor			
			I: III, Chu-Green method (5)	III, Schwartz fluorescence quenching method (12)	
	°C.	γ		*per cent*	γ
1	234–244	156	III	92	143
2	238–245	184	I > III	43	79
3	242–247	140	I > III	63	88
Ether wash		104	I > III	24	25
4	246–248	20	I	10	2
Final crystallization		2100		6	126
		2704			463 or 15% of total

An x-ray crystallographic study was carried out on the final crystals. The pattern was identical with that of coproporphyrin I.

dilute ammonia. The eluate was neutralized with HCl, a small amount of sodium acetate was added, then 0.5 volume of acetone, after which the porphyrin was adsorbed on Al_2O_3 and eluted and esterified with methyl alcohol-H_2SO_4 (19:1). From this point the methyl ester was handled in the usual way, crystallizing readily from $CHCl_3$-CH_3OH (1.43 mg.). Both ester fractions behaved as entities (uroporphyrin) by MgO chromatography. By Falk-Benson chromatography about 30 per cent of the total ran as uroporphyrin I in the first instance and about 20 per cent in the second, despite the known composition of 80 per cent type I in both. It was again significant that the only spots observed corresponded with those of uroporphyrins I and III; that which characterizes the "208" porphyrin either alone or in a simple mixture with uroporphyrin I ester in $CHCl_3$ failed to appear. The crystals of the major fraction (ethyl acetate) melted

at 258–260° after three and four recrystallizations; those of the smaller fraction (talc) melted at 265–270° also after three and four recrystallizations. 1.2 mg. of the former and 1.1 mg. of the latter were decarboxylated, yielding 0.78 and 0.5 mg. of coproporphyrin methyl ester, respectively. Both crystallized readily, very little porphyrin remaining in the mother liquor. The total coproporphyrin ester from the ethyl acetate fraction (0.78 mg.) behaved mainly as type I, with a minority of type III, on Chu-Green chromatography; by the fluorescence quenching method of Schwartz *et al.* (12), there was 30 per cent type III in this fraction. This value significantly exceeds the theory of 20 per cent; *i.e.*, the proportion of "208" porphyrin in the original mixture. It is recognized, however, that this method permits only a rough approximation. The melting point after the first crystallization was 232–240°. Very little porphyrin remained in the mother liquor. The total coproporphyrin from the talc fraction (see above) amounted to 0.5 mg. After one recrystallization the melting point was 242–246°. The total mother liquor contained but 0.042 mg.

The uroporphyrin esters from both experiments were subjected to x-ray crystal powder pattern analysis.[3] The interplanar spacings are presented in Table II. They may be compared with those of naturally occurring Waldenström porphyrins as well as with uroporphyrin I and III and the "208" porphyrin.

The following experiments served as further controls for the above studies.

Experiment 3a—10 mg. of uroporphyrin I methyl ester, m.p. 288–292°, were saponified by standing overnight in 7.5 N HCl. After neutralization with NaOH and $NaOOCCH_3$, the solution (25 ml.) was slightly alkalized with Na_2CO_3. This was mixed with 75 ml. of fresh normal urine, final pH about 8.0. The mixture was refrigerated at 4° overnight, then acidified with 3.0 N HCl to a gray-blue Congo paper reaction (pH 3.2). From this point on the treatment was exactly as in Experiment 2. The total uroporphyrin extracted by ethyl acetate amounted to but 0.31 mg.; that from the subsequent talc adsorption was 4.38 mg. The crystalline methyl ester obtained by ethyl acetate extraction melted at 290–294° and behaved entirely as uroporphyrin I by the Falk-Benson method; that from the subsequent talc adsorption melted from 287–292°, unchanged after four recrystallizations. This also behaved as uroporphyrin I by Falk-Benson chromatography.

[3] We are indebted to Professor William Lipscomb, Division of Physical Chemistry, University of Minnesota, for use of the powder camera, also for valued advice and assistance. The following apparatus was used: powder camera from the Central Research Laboratories, Red Wing, Minnesota (diameter 17.95 cm. for 180°); x-ray unit from General Electric.

Experiment 3b—Several mixtures of uroporphyrin I ester (288–292°) and the "208" porphyrin ester (206–208°) were mixed in varying proportions in

TABLE II

x-Ray Crystal Powder Patterns

Angle measured in cm.

Uroporphyrin I	Uroporphyrin III*	7-COOH "208"	Waldenström porphyrin, natural			Waldenström porphyrin, artificial	
			Type A†	Gray, Type B	Type A†	Type B, Experiment 1	Type B, Experiment 2
						3.74 M.	
						3.37 W.	3.11 W.
		2.67 W.					
2.51 W.	2.58 W.			2.56 W.	2.58 W.	2.55 M.	
2.34 "		2.37 "				2.41 W.	
		2.29 "					
2.13 "	2.21 M.	2.20 "	2.20 W.	2.19 "	2.21 M.	2.19 M.	2.13 M.
2.02 "		1.98 "	2.00 "		1.97 W.	2.01 W.	
						1.95 "	
		1.80 "				1.80 M.	
1.64 "	1.69 "	1.66 "	1.55 "	1.62 "		1.61 W.	
1.47 "	1.43 "	1.47 M.			1.47 "	1.46 "	
1.40 M.		1.33 W.	1.38 "	1.39 "	1.36 "	1.39 "	1.37 W.
1.29 "		1.31 M.		1.29 "		1.33 "	1.32 "
1.21 "	1.22 "				1.25 "	1.25 "	
1.15 "	1.14 W.	1.15 "	1.19 M.	1.20 "	1.19 M.	1.18 M.	1.19 "
	1.09 M.		1.13 W.		1.15 W.	1.13 W.	1.14 "
1.07 S.	1.06 S.	1.07 S.	1.07 S.	1.07 S.	1.07 S.	1.06 S.	1.06 S.
	1.01 W.	1.00 "	1.00 W.	1.04 M.	1.00 W.		
0.99 M.				0.99 "		0.99 W.	0.98 W.
	0.95 S.					0.96 "	
0.92 "	0.91 W.	0.92 M.	0.93 M.	0.94 S.	0.94 S.	0.90 "	0.91 M.
0.84 "	0.85 S.	0.84 "	0.84 "	0.85 "	0.85 "	0.82 "	0.83 W.
0.76 W.	0.76 W.		0.76 W.	0.77 W.	0.77 W.	0.75 "	0.75 "
0.69 "							
0.61 "						0.60 "	
0.55 "	0.54 "	0.53 W.	0.53 "	0.54 "	0.54 "	0.54 "	

M. = medium; W. = weak; S. = strong.

* From turacin, provided through the courtesy of Professor Rimington.

† Individual isolations from different cases of porphyria.

order to observe resolution by MgO and $CaCO_3$ chromatography without prior treatment with urine. The mixture was made in $CHCl_3$ solution, which was then taken to dryness under reduced pressure. The residue was subjected in each instance first to MgO (10) then to $CaCO_3$ chroma-

tography (2) (Table III). The mixtures and results with $CaCO_3$ are presented in Table III. No resolution was observed with MgO.

Experiment 3c—To control the possible effects of concentration under reduced pressure, mild heating, and saponification of esters with strong HCl, 4.2 mg. of the same uroporphyrin I methyl ester and 1.05 mg. of "208" porphyrin ester of the same source were mixed in $CHCl_3$ solution and evacuated to dryness with gentle heating in the water bath under reduced pressure. The mixed ester residue was dissolved in 7.5 N HCl and allowed to stand overnight. The pH was then adjusted to approximately 3.2 (gray-blue to Congo paper) and the solution extracted repeatedly with ethyl acetate. This contained 0.88 mg. of a free uro type porphyrin, while 3.45 mg. of free porphyrin were obtained from the aqueous phase. After reesterification, the former was crystallized with ester, melting point of 203–206°, the latter of 278–282°. While these melting points indicate that resolution by the ethyl acetate extraction was not complete, it is evident that this mixture behaved quite differently toward ethyl acetate than in the experiments with urine (Experiments 1 and 2).

TABLE III

Resolution of Mixtures of Uroporphyrin I and "208" Esters by $CaCO_3$ Chromatography

Sample No.		Zones on $CaCO_3$	M.p.
			°C.
1	1.6 mg. uroporphyrin I + 0.4 mg. "208" porphyrin ester	1	282–285
		2	206–208 (0.31 mg.)
2	1.0 mg. uroporphyrin I + 1.0 mg. "208"	1	284–288
		2	202–207 (0.85 mg.)
3	0.2 mg. uroporphyrin I + 1.0 mg. "208"	1	282–288
		2	204–206 (0.9 mg.)

DISCUSSION

From the above data it is clear that a molecular association of 80 per cent uroporphyrin I and 20 per cent "208" porphyrin (type III, heptacarboxyl), similar to that previously isolated from acute porphyria urine (2, 3), is likely to be confused with uroporphyrin III (from turacin), on the basis of Falk-Benson chromatography. It is believed significant that a mixture of these porphyrins, in the ratio mentioned, on standing in normal urine provides an association behaving as an entity, or so called "type B Waldenström" porphyrin. After repeated recrystallization a constant ester melting point is observed such as characterizes the naturally occurring Waldenström porphyrin ester. Control studies showed, however, that simple mixtures in

the same and in varying proportion, not exposed to urine, were readily resolved on $CaCO_3$, but not on MgO. These and other studies have made it fully evident that the latter adsorbent cannot be expected to resolve either mixtures or molecular associations of uroporphyrin I and the "208" porphyrin, but that a suitable $CaCO_3$, *i.e.* one with an iodine number of 170 to 180, will readily effect this separation. This is believed to account for the failure of others to isolate the "208" porphyrin ester. Similarly, paper chromatography with lutidine-water (13), carried out at 23° with 2,6- and 2,4-lutidine descending (24 hours) as well as ascending (5 hours) and with the mixtures suggested by Erikson (14), did not resolve the Waldenström complex, either A or B, although the A type is readily resolved by use of a suitable $CaCO_3$. The behavior of the A type on Falk-Benson chromatography is quite variable. Further studies bearing on this point are described in a separate communication.

The differing x-ray crystal powder patterns are of considerable interest. The fact that differences are found not only between the "artificial" and naturally occurring Waldenström esters, but among the latter as well, indicates that the term "Waldenström porphyrin" refers to a group of molecular associations rather than to any sharply defined entity. That minor differences may occasion significant variations in powder pattern was emphasized by the differing patterns for the Gray porphyrin ester following $CaCO_3$ as against MgO purification described in Paper IV. In Table II it is seen that the pattern obtained from the artificial Waldenström ester of Experiment 2 was not as clearly defined as that of Experiment 1. This has been true on several repetitions, and the reason for the difference is not clear. It may be noted, however, that the pattern in Experiment 2 is essentially as clear as that of the standard uroporphyrin III obtained from Professor Rimington. Kennard and Rimington (15) have shown that the powder patterns of simple mixtures of the uroporphyrins (I and III) are quite diffuse over a considerable range of varying proportions. In the case of the present artificial Waldenström esters a molecular complex is assumed, both because of the relatively sharp powder patterns, especially with that obtained in Experiment 1, together with the behavior as an entity on $CaCO_3$ and the constant melting point on repeated recrystallization. It has also become evident in recent studies that, at least as exemplified by coproporphyrins I and III, isomers themselves may associate or form complexes and crystallize in a manner productive of a sharp powder pattern differing from that observed with a simple mixture of the crystalline esters.

Since completion of the above studies a paper has appeared by Dresel and Tooth (16) in which evidence is presented that under certain specific conditions uroporphyrin I may be extracted quantitatively from aqueous

solution by ethyl acetate. It is not stated whether this method permits extraction from urine. In the present study the conditions at least closely simulated those of Waldenström *et al.* (1) and the results were essentially similar; in other words, uroporphyrin I was poorly extracted, while the Waldenström type porphyrin was efficiently removed by ethyl acetate. It may also be noted that in Experiment 3c ethyl acetate failed to extract uroporphyrin I efficiently from an aqueous solution in the absence of urine.

SUMMARY

1. A mixture of 4 parts of uroporphyrin I with 1 part of the "208" (type III) porphyrin, dissolved and allowed to stand in normal urine, behaves as a single or type B Waldenström porphyrin, such as is often obtained from acute porphyria urine.

2. The complex thus formed is largely extracted from the urine at pH 3.2 by ethyl acetate, in accord with Waldenström's observation and in contradistinction to uroporphyrin I.

3. The complex isolated as the crystalline methyl ester has the melting point characteristic of the natural Waldenström ester, 258–260°.

4. The complex behaves as an entity both by $CaCO_3$ and MgO chromatography. A simple mixture of uroporphyrin I and the "208" porphyrin esters in the same proportions in $CHCl_3$ likewise behaves as a single uroporphyrin by MgO chromatography, although readily separable on $CaCO_3$ or by the Falk-Benson paper method.

5. The artificial Waldenström ester, although known to be composed of 80 per cent uroporphyrin I, behaves mainly as a type III uroporphyrin by the Falk-Benson method. No spot for the "208" porphyrin is observed. This is also true for lutidine-water paper chromatography.

6. Semiquantitative decarboxylation of the artificial Waldenström porphyrin and study of the resulting coproporphyrins confirm the value of this method in determining the isomer ratio of molecular associations.

7. X-ray crystal powder patterns reveal distinct differences between artificial and natural Waldenström esters, and significant variations are observed among the latter, even in relation to the method of purification of the same ester.

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A CHEMICAL COMPARISON OF THREE STRAINS OF TOMATO BUSHY STUNT VIRUS*

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Chemical analyses of thirteen different strains of tobacco mosaic virus (TMV) demonstrated that the nucleic acid compositions were essentially identical in all strains studied, though distinctly different from those of all other plant viruses that were investigated (1–3). However, the protein composition was shown to vary from strain to strain, with the exception of three pairs of strains which appeared to have identical amino acid content.

These studies of TMV indicated that the mutation of a virus might be accompanied by a measurable change in the amino acid composition, while the nucleic acid composition remained unchanged. It was considered desirable to investigate an entirely different plant virus to see whether the same strain relationship was observed. For this purpose, several recently described, closely related strains of tomato bushy stunt virus (BSV) (4) were selected. The three strains were selected on the basis of their widely differing effects on the plant, *Datura stramonium*. These strains were cultured, purified, and analyzed for their protein and nucleic acid composition. The results of the analyses are reported herein.

Materials and Methods

Mutant Strains—The original inocula of the three strains were obtained from Dr. R. L. Steere, who had isolated them by repeated passage through local lesions in *Nicotiana glutinosa*. A brief description of the effects of the three strains on the host plant, *D. stramonium*, is given below.

Strain BS3—This strain causes an appreciable stunting of the plants. In addition, the leaves appear to be blistered and show a mottled discoloration, light greenish to yellow.

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Strain BS9—This strain is characterized by a pronounced chlorosis of the leaves which present brilliant patches of yellow. The plant, however, is not dwarfed; nor are the leaves disfigured.

Strain BS10—This is perhaps the mildest of all the strains isolated. Infected leaves show slightly darker green patches. As in BS9, there is no stunting or leaf distortion.

The viruses were purified by the method of Stanley (5) with the following modifications: The freshly harvested plants were worked up immediately, instead of being frozen. The plants were minced in a commercial food cutter[1] rather than in a meat grinder. In no step of the purification was any salt added to the preparation; nor was refrigeration used for any of the centrifugal separations. BS3 gave up to 1.2 gm. of dried virus per liter of expressed sap, while infection with the other two strains resulted in much lower yields, less than 300 mg. on the same basis.

Nucleic Acid Analysis—Phosphorus analyses were carried out by the method of Nakamura (6). When analyses were to be performed on dried virus preparations, the virus was dissolved directly in 60 per cent perchloric acid at an appropriate concentration. In order to provide a basis for expressing results in terms of moles base per mole of phosphorus, phosphorus determinations were also made on aliquots of the virus hydrolysates for nucleotide analyses.

The method used for the determination of the various nucleotides in the nucleic acid was that of Smith and Markham (7). Since Dorner and Knight (3) have shown that nucleic acid analyses of the whole virus give essentially the same results as analyses of the isolated nucleic acid, determinations were carried out with the unfractionated material. A correction was employed for the slight amount of hydrolysis of cytidylic and uridylic acids to cytidine and uridine, respectively. In the chromatographic system used (*t*-butanol-HCl-water), the cytidine spot coincided with that of adenine, while uridine migrated to a position intermediate between cytidylic acid and uridylic acid. In standard hydrolysates, however, no uridine spot could ever be detected by visual observation. On the other hand, a calculation of the 260:280 ratio of the adenine spot indicated that there was a small but definite contribution to the light absorption by cytidine. The amount of cytidine was 3 to 5 per cent of the total amount of cytidylic acid. The exact amount was calculated by the use of simultaneous equations with the observed optical densities at 260 and 280 mμ, and the extinction coefficients of standard nucleotides run simultaneously.

The purine-bound pentose of each strain was qualitatively identified as

[1] Hobart food cutter model T125.

ribose by paper chromatographic methods, as previously described (3), with aliquots of the same hydrolysates employed for determination of the nucleotide compositions.

Protein Analysis—The protein moiety of the virus was analyzed by the method of Moore and Stein (8). The virus samples were hydrolyzed in sealed tubes *in vacuo* with 200 times their weight of glass-distilled, constant boiling hydrochloric acid for 17 hours at a temperature of 112–115°. All the samples were hydrolyzed at the same time to minimize variations in the conditions of hydrolysis. As the results were to be expressed as micrograms of amino acid per microgram of phosphorus, an aliquot of each hydrolysate was removed for phosphorus analysis.

Two controls were subjected to the hydrolysis conditions at the same time that the samples were being hydrolyzed. The first was a "hydrolysis" control, designed to measure the extent of decomposition occurring to

TABLE I

Phosphorus Content of Some Mutants of Tomato Bushy Stunt Virus

Strain No.	No. of determinations	Per cent P	S.d.*
BS3	20	1.58	0.030
BS9	19	1.58	0.051
BS10	19	1.61	0.029

* Standard deviation of the distribution.

the individual amino acids during hydrolysis. It was prepared by dissolving an accurately weighed amount of each amino acid to be analyzed in glass-distilled, constant boiling hydrochloric acid. Since the hydrolytic losses of amino acids are increased in the presence of carbohydrate material (9), a small amount of ribose, the naturally occurring pentose of bushy stunt virus nucleic acid, was included. The second control was designed to correct for the amount of glycine which may arise during strong acid hydrolysis of nucleic acid-containing compounds, as has been discussed by Smith and Markham (7). It was prepared by dissolving a mixture of nucleotides (in the same proportions as they occur in bushy stunt virus nucleic acid) in glass-distilled, constant boiling hydrochloric acid.

The elution and photometric analysis were carried out as described by Moore and Stein (8, 10), with only one modification. This concerned the small peak which appears before aspartic acid. The color value of this peak, which has been identified by Schram *et al.* (11) as methionine sulfoxide, was determined, and the calculated methionine equivalent was

added to the methionine value of the normal peak appearing just before isoleucine.

Since the results of the arginine determination by the chromatographic method were not reproducible, arginine was analyzed by the method of

TABLE II

*Nucleic Acid Compositions of Some Mutants of Tomato Bushy Stunt Virus**

Base	Strain No.			S.d.†
	BS3	BS9	BS10	
Adenine	0.263	0.260	0.259	0.0031
Guanine	0.286	0.284	0.282	0.0062
Cytosine	0.212	0.208	0.205	0.0060
Uracil	0.263	0.258	0.257	0.0051
Sum	1.024	1.010	1.003	

* The results are expressed in terms of the moles of base per mole of phosphorus.
† Standard deviation of the distribution.

TABLE III

*Amino Acid Composition of Some Mutants of Tomato Bushy Stunt Virus**

Amino acid	Strain No.			S.d.†	Amino acid	Strain No.			S.d.†
	BS3	BS9	BS10			BS3	BS9	BS10	
Alanine	3.70	3.87	3.76	0.120	Lysine	2.21	2.24	2.25	0.087
Aspartic acid	6.70	6.94	6.70	0.108	Methionine	0.52	0.65	0.71	0.035
Cystine	0.0	0.0	0.0		Phenylalanine	2.62	2.78	2.61	0.112
Glutamic acid	3.58	3.65	3.49	0.112	Proline	2.08	2.00	2.02	0.092
Glycine	3.34	3.49	3.37	0.125	Serine	4.23	4.04	4.14	0.142
Histidine	0.88	0.95	0.84	0.080	Threonine	6.03	6.08	5.95	0.150
Isoleucine	1.97	2.16	1.98	0.072	Tyrosine	2.14	2.35	2.29	0.117
Leucine	6.52	6.65	6.51	0.127	Valine	5.40	5.22	5.33	0.191

* The results are expressed in terms of micrograms of amino acid per microgram of phosphorus.
† Standard deviation of the distribution.

Sakaguchi (12). Amide nitrogen was determined by the method of Fraenkel-Conrat and Olcott (13). A correction was applied for the small amount of ammonia which arose from the deamination of the nucleotide, cytidylic acid. Tryptophan was determined in the intact virus by the method of Spies and Chambers (14), and cysteine was determined in the native virus by the method of Tsao and Bailey (15).

Results

The results of the phosphorus and nucleic acid analyses are shown in Tables I and II, respectively. As indicated in Table II, the sum of the

TABLE IV

*Constituents of Some Mutants of Tomato Bushy Stunt Virus Determined by Methods Other Than Column Chromatography**

Constituent	Strain No.			S.d.†
	BS3	BS9	BS10	
Arginine	6.35	6.53	6.34	0.224
Cysteine	0.74	0.71	0.75	0.025
Tryptophan	0.67	0.65	0.65	0.018
Amide nitrogen	1.17	1.16	1.15	0.020

* The results are expressed in terms of per cent dry weight.
† Standard deviation of the distribution.

TABLE V

*Amino Acid Composition of Some Mutants of Tomato Bushy Stunt Virus**

Amino acid	Strain No.			Amino acid	Strain No.		
	BS3	BS9	BS10		BS3	BS9	BS10
Alanine	4.7	4.9	4.8	Lysine	3.1	3.1	3.1
Arginine	5.7	5.9	5.7	Methionine	0.7	0.9	1.0
Aspartic acid	9.2	9.5	9.2	Phenylalanine	3.7	3.9	3.7
Cysteine	0.6	0.6	0.6	Proline	2.8	2.7	2.7
Glutamic acid	5.0	5.1	4.9	Serine	5.6	5.3	5.5
Glycine	4.0	4.2	4.1	Threonine	8.1	8.2	8.0
Histidine	1.2	1.3	1.2	Tryptophan	0.6	0.6	0.6
Isoleucine	2.7	3.0	2.7	Tyrosine	3.1	3.4	3.3
Leucine	8.9	9.1	8.9	Valine	7.3	7.0	7.2
Sum					77.0	78.7	77.2

* The results are expressed in terms of gm. of amino acid residue per 100 gm. of virus.

purine and pyrimidine bases is in all cases equal to the amount of phosphorus present. Thus, it is apparent that there is no extensive decomposition of the bases during hydrolysis.

The results of the amino acid analyses obtained by the Moore and Stein technique are presented in Table III. The "hydrolysis" control discussed

above suggested that the only amino acids which were unstable under the hydrolytic conditions employed (in addition to tryptophan) were serine, threonine, and tyrosine. These three amino acids were destroyed to the extent of 13.6, 6.5, and 6.0 per cent, respectively. The correction for the formation of glycine from the decomposition of nucleic acid reduced the apparent glycine content by 9 per cent.

The results of the arginine, cysteine, tryptophan, and amide nitrogen determinations are presented in Table IV. The correction which was applied to the amide nitrogen determination amounted to approximately 6 per cent of the true amide nitrogen content.

For purposes of comparison, the data of Tables III and IV were recalculated as gm. of amino acid residue per 100 gm. of virus, and are presented in Table V. The data of Table III were recalculated with the average phosphorus content found for the three strains of BSV; namely, 1.59 per cent.

DISCUSSION

An examination of Table I shows that, with respect to the phosphorus content, the samples are identical within analytical limits. Since the phosphorus content of a plant virus is indicative of the amount of nucleic acid present, it is apparent that the three strains of BSV contain similar, if not identical, amounts of nucleic acid. According to the data of Table II and the average phosphorus content found for the three strains of BSV this amount is 16.5 per cent.

From Table II, it can be seen that the amount of any particular nucleotide within the nucleic acid is essentially the same in all strains. If differences do exist in the nucleic acid composition, they must lie below the limits of the present methods of analysis (± 2 per cent). On the other hand, it is possible that the nucleotide compositions of the strains are identical, but that the sequence of the nucleotides or even the spatial arrangement of the nucleotide chain, or chains, is different from strain to strain.

The data of Table III reveal a striking similarity in the compositions of the proteins of the three strains studied. While a statistical analysis, with Fisher's t test (16), indicates that BS9 contains significantly higher amounts of aspartic acid, histidine, and isoleucine than the other two strains, these differences are just significant at the 5 per cent confidence level. Since a significant difference at the 5 per cent confidence level means that there is a 1 in 20 chance that this difference is due to sampling variation alone, and since the amino acids determined represent eighteen variables for each sample, it is clear that a 5 per cent confidence level is not sufficient to establish the presence of real differences. Another point which

suggests that the slight differences between BS9 and the other two strains may be apparent rather than real is the fact that the summation of the amino acid residues is greater in BS9 than in either of the other strains. This discrepancy, although amounting to only a 2 per cent difference, reflects in large part the generally higher amino acid values of BS9. A consideration of these arguments leads one to conclude that the amino acid compositions of the three strains of BSV, if not identical, are very similar.

A summation of the amino acid data, as in Table V, indicates a recovery of 92 to 94 per cent of the protein components in the form of amino acid residues. This low recovery can perhaps be explained on the basis that the "hydrolysis" control did not account for the decomposition of the individual amino acids during release from peptide linkages. As this loss cannot be determined conveniently, the "hydrolysis" control can be considered to provide only a minimal correction.

If one calculates from Tables III and IV the average molar ratio of aspartic acid, glutamic acid, and amide nitrogen in some arbitrary units (*e.g.*, micromoles per microgram of phosphorus), the values are 0.0509, 0.0243, and 0.0521, respectively. The close similarity in the amounts of aspartic acid and amide nitrogen might be mere coincidence in an isolated case, but the fact that Knight has observed an identical situation in the case of several strains of TMV (17) raises the interesting possibility that the virus structure may contain the acidic amino acids in the form of asparaginyl and glutamyl residues exclusively.

The results of the present study on the chemical composition of three strains of BSV demonstrate a striking similarity in constitution between mutants of a virus possessing quite different biological effects. In this respect, the results agree with those found with some of the strains of TMV between which no chemical differences were apparent. It now appears that the differences between protein compositions found for some strains of TMV are of secondary importance and that the vital distinction between viral strains must lie in structural features yet to be defined.

The authors are indebted to Dr. Dean Fraser for assistance and helpful suggestions in the ion exchange column chromatography, and to Dr. H. Fraenkel-Conrat for collaboration on the cysteine determinations.

SUMMARY

Three strains of tomato bushy stunt virus (BSV), which were selected for their widely differing effects on the plant, *Datura stramonium*, were highly purified and analyzed for their nucleic acid and protein composition.

The content of nucleic acid, about 16.5 per cent, and its nucleotide com-

position were found to be the same for all strains; likewise, the purine-bound pentose was identified in each case as ribose.

The protein moieties were analyzed by the Moore and Stein technique of ion exchange column chromatography supplemented by colorimetric procedures. No decisive differences were found, and hence it was concluded that the amino acid compositions of the three strains are very similar if not identical.

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FAT METABOLISM IN HIGHER PLANTS

V. A SOLUBLE PALMITATE OXIDASE REQUIRING GLYCOLIC ACID AS ACTIVATOR

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In previous papers in this series (1) it was reported that microsomal particles obtained from cotyledons of germinated peanuts catalyze the oxidation of palmitic acid. Although no cofactor requirements were demonstrable for $C^{14}O_2$ production from palmitic acid-1-C^{14}, DPN[1] is specifically required for $C^{14}O_2$ production from palmitic acid-2-C^{14} and -3-C^{14}. Synthetic palmityl-1-C^{14} CoA is inactive. Furthermore, the tricarboxylic acid cycle is not involved in CO_2 formation from palmitate oxidation.

This communication will present the results of experiments with the supernatant fraction of peanut homogenates, freed of mitochondria and microsomes by differential centrifugation. This system catalyzes a limited oxidative decarboxylation of palmitic acid-1-C^{14} to $C^{14}O_2$ and an unidentified fragment. Internally labeled palmitic acid is inert. ATP, CoA, DPN^+, and Mg^{++}, as well as other cofactors, do not participate in the system. However, substrate concentrations of either glycolic acid or L-lactic acid are required for the reaction to proceed.

EXPERIMENTAL

Preparation of S_{AS}—The procedure for obtaining the supernatant enzyme (S_{AS}) by differential centrifugation of peanut homogenates has been described by Humphreys *et al.* (1). The enzyme system is freed of small molecular weight compounds such as cofactors, etc., by precipitating the original supernatant fraction with ammonium sulfate.

4 volumes of ice-cold, saturated ammonium sulfate solution (adjusted to pH 7.0) were added with constant stirring to 1 volume of crude supernatant

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[1] The following contractions are used: DPN, diphosphopyridine nucleotide; CoA, coenzyme A; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; AMP, adenosine monophosphate; UTP, uridine triphosphate, GTP, guanosine triphosphate; CTP, cytosine triphosphate; GSH, glutathione; FAD, flavin adenine dinucleotide; TPP, thiamine pyrophosphate; GDP, guanosine diphosphate; ITP, inosine triphosphate; TPN, triphosphopyridine nucleotide.

fraction. The suspension of insoluble proteins was centrifuged for 5 minutes at 13,000 × *g* at 4°. The white pellet was resuspended in 2 volumes of cold 0.8 saturated ammonium sulfate and the fine suspension was again centrifuged for 5 minutes. The pellet was dissolved in 1 volume of 0.2 M Tris-0.2 M KCl buffer at pH 7.2. This preparation can be stored at −10° for several weeks with little loss in activity. Exposure to 60° for 5 minutes at pH 7.0 completely inactivates the enzyme.

Partial Resolution of S_{AS}—Partially resolved enzyme was prepared by passing 10 ml. of S_{AS} through a Dowex 1-Cl^- column 12 mm. in diameter containing 10 gm. of moist resin. The operation was carried out at 5°. The eluate was treated batchwise with 25 mg. of charcoal (Darco S-51) per ml. of protein solution for 20 minutes at 0°. The charcoal is then centrifuged. This enzyme preparation is designated as S_{ASR}; in contrast to S_{AS} this preparation cannot be stored at −10° without considerable loss in activity.

Substrates—Palmitic acid-1-C^{14} was obtained from Tracerlab, Inc., Boston, and stearic, lauric, and myristic acids-1-C^{14} were obtained from Professor I. Chaikoff, Department of Physiology, University of California. L-Lactic acid-1-C^{14} was obtained from Professor H. A. Barker, Department of Plant Biochemistry, University of California, and glycolic acid-1-C^{14} from Dr. Andrew Benson, Radiation Laboratory, University of California.

α-Hydroxypalmitic acid-1-C^{14} was prepared by the bromination of palmitic acid-1-C^{14} and hydrolysis of the α-bromo acid with potassium hydroxide in 30 per cent ethanol; m.p. 86–88°; reported m.p. 86–87° (2).

α-Ketopalmitic acid-1-C^{14} was prepared from α-hydroxypalmitic acid-1-C^{14} by esterifying the hydroxy acid with diazomethane, oxidizing the methyl ester with chromic acid in glacial acetic acid at 35°, and gently saponifying the ester with potassium hydroxide in 30 per cent ethanol; m.p. 65–67°; previously reported m.p. 70–71° (3).

α-Hydroxypalmitic and α-ketopalmitic acids were dissolved in water and neutralized with 0.1 N ammonium hydroxide to give a final concentration of 0.001 M solutions.

Reagents—0.001 M palmitic acid. Sufficient radioactive palmitic acid (approximately 10^5 c.p.m.) was added to carrier palmitic acid to make a total of 2.56 mg. of substrate which was suspended in 5 ml. of distilled water. The suspension was then heated to boiling and 1 M NH_4OH was added dropwise until the fatty acid was dissolved. The solution was diluted to a final volume of 10 ml. and stored at −5°. The palmitate solution was always heated to 100° to bring the substrate into complete solution and then added to the reaction mixtures in required amounts.

0.2 M Tris-0.2 M KCl. 0.2 M Tris buffer was adjusted to pH 7.2 with 6 N HCl. To 100 ml. of buffer were added 1.5 gm. of KCl.

20 per cent KOH. KOH is dissolved in boiled distilled water.

25 per cent barium acetate. The salt is dissolved in boiled distilled water.

Reaction Mixture—To study the reaction

$$\text{Palmitic acid-1-C}^{14} \rightarrow x + \text{C}^{14}\text{O}_2$$

each Warburg flask contained 0.1 μmole of palmitic acid-1-C^{14}, 0.5 ml. of S_{AS}, varying concentrations of cofactors, inhibitors as indicated, 0.5 ml. of Tris-0.2 M KCl buffer at pH 7.2, and water to a total volume of 3.0 ml. In the side arm was added 0.3 ml. of 5 N sulfuric acid and in the center well 0.2 ml. of 20 per cent KOH. The reaction was permitted to go for 1 hour at 30°, at the end of which time acid was tipped in from the side arm to stop the reaction and release bound $C^{14}O_2$. Shaking was continued for 10 minutes, after which the contents of the center well were removed quantitatively. The respiratory CO_2, precipitated as $BaCO_3$ by the addition of 0.3 ml. of 25 per cent barium acetate, was washed three times with 10 ml. of 50 per cent ethanol, and the precipitate was then plated on aluminum disks and counted with a thin window Geiger-Müller tube.

Results

Cofactor Requirements—When palmitic acid-1-C^{14} is added to the crude supernatant fraction, a rapid release of $C^{14}O_2$ is observed. If this preparation is brought to 0.8 saturation with respect to ammonium sulfate, and the precipitate is collected and redissolved (S_{AS}), the ability to oxidize palmitic acid-1-C^{14} is lost. As summarized in Table I, this ability can be completely restored by the addition of boiled crude enzyme. The addition of AMP, ATP, UTP, GTP, CTP, GSH, CoA, DPN, TPN, cytochrome *c*, FAD, folic acid, biotin, lipoic acid, lipothiamide pyrophosphate, TPP, Mg^{++}, Mn^{++}, and Krebs cycle intermediates alone or in combination failed to replace the factor associated with boiled crude enzyme preparations.

On examining the properties of this factor, it was observed that it is stable to acid or alkaline conditions (7 minutes in 0.1 N alkali or acid at 100°), is adsorbed on Dowex 1-Cl^- but not on Dowex 50-K^+, nor on charcoal (Darco S-51), and is extracted by ether under acid conditions in a liquid-liquid extractor (24 hours of continuous extraction required). When these properties were employed as the basis for the development of an extraction procedure, a concentrate of the cofactor was obtained from 2 liters of peanut homogenate prepared from 800 gm. of germinated peanut cotyledons. Cofactor activity was followed by testing each stage of fractionation with the S_{AS}-palmitate-1-C^{14} system.

Since the active component had the properties of a non-volatile, small molecular weight acid with no nucleotide properties, the final identification

was made by using paper chromatographic methods developed for the separation of short chain organic acids.

Peanut concentrate was chromatographed one-dimensionally with *n*-butanol-formic acid as the developing solvent (4). The paper was dried, then sprayed with an acid indicator (0.04 per cent bromocresol green in 95 per cent alcohol adjusted to blue-green with 1 N KOH). Three acidic bands appeared with R_F values corresponding to 0.5, 0.7, and 0.8. The entire paper was cut into strips 1 cm. wide and parallel to the origin, with the exception of the acidic bands which were cut out carefully. The strips were eluted with small volumes of water and the eluates were tested for activation effects. All strips were found inactive, with the exception of the acidic bands corresponding to the R_F values of 0.5 and 0.7. In order to identify the unknown bands, a series of known acids was chromatographed under the same conditions. It was found that only glycolic acid

TABLE I

Activation Effect for Oxidation of Palmitic Acid-1-C^{14}

See under "Experimental" for assay.

Enzyme system	Per cent $C^{14}O_2$ as $BaC^{14}O_3$
1. Crude supernatant fraction	27
2. S_{AS} fraction	0
3. " + boiled crude supernatant fraction	26

and lactic acid had R_F values similar to those of the unknown bands. When pure glycolic acid and L-lactic acid were tested for activation effects, they completely replaced the peanut concentrate. An analysis of the concentrate showed that 25 per cent of the total weight was lactic acid and 3 per cent glycolic acid.

In order to rule out the possibility that activation may be caused by some impurity present in low concentrations in laboratory grade lactic acid, highly purified sodium pyruvate was reduced to DL-lactic acid with sodium borohydride by the method of Reid and Siegel (5). The reduction product completely replaced L-lactic acid.

Specificity of Activation—The finding that either glycolic acid or L-lactic acid participates in some manner in the release of $C^{14}O_2$ from palmitic acid-1-C^{14} is not easily explained on the basis of known mechanisms for fatty acid oxidation. In order to clarify the specificity requirements of the activation, a large number of related compounds were tested and the results summarized in Table II. The data indicate that (*a*) glycolic acid is the most active, (*b*) in general, L-α-hydroxy aliphatic acids may serve as activators in higher concentrations, (*c*) D isomers are completely inert and

do not inhibit the system, (*d*) α-keto acids and β-hydroxy and β-keto acids are inactive, and (*e*) Krebs cycle acids, α,β-unsaturated acids, alanine, serine, and glycine do not activate or inhibit the system.

Stability of α-Hydroxy Acids—The observation that either glycolic acid or L-lactic acid participates in the oxidation of palmitate by the S_{AS} system would immediately suggest that these hydroxy acids undergo changes during the course of the reaction. However, when glycolic acid-1-C^{14} and -2-C^{14} and DL-lactic acid-1-C^{14} were incubated with S_{AS} in either the presence or the absence of palmitic acid, no radioactivity was found in the respiratory CO_2. This result would suggest the absence of glycolic oxidase. In addition, paper chromatographic examination of these reaction mixtures for acidic derivatives such as acetic acid, oxalic acid, and formic acid did not reveal any new spots which could have resulted from an interaction of the hydroxy acids with palmitic acid. It is possible that these α-hydroxy acids, by participating in a cyclic process, are never degraded by the S_{AS} system.

TABLE II

Activation of Oxidative System by Various Carboxylic Acids

Activator	Final concentration	Per cent total $C^{14}O_2$ as $BaC^{14}O_3$
	μmoles	
None		0
Glycolic acid	50	51
" "	10	50
" "	1	32
Glyoxylic acid	50	0
L-Lactic acid	50	52
" "	10	9
D-Lactic acid	50	0
DL-Lactic acid	50	50
Pyruvic acid	50	0
DL-α-Hydroxybutyric acid	50	40
DL-β-Hydroxybutyric "	50	0
α-Ketobutyric acid	50	0
DL-α-Hydroxyvaleric acid	50	35
DL-Phenyllactic acid	50	0
Acrylic, crotonic, levulinic, tartronic, L-malic, citric, fumaric acids, glycine, alanine, serine	50	0

pH Effects—The effect of pH on the liberation of $C^{14}O_2$ from palmitic acid-1-C^{14} was studied with 0.06 M Veronal buffer over the range pH 4.5 to 11.0. Reactions were run in the presence of either glycolic acid or L-lactic

acid and in both instances the pH optima occurred as a rather broad plateau between pH 6 to 7.5.

Reaction As Function of Time—The effect of time on the release of $C^{14}O_2$ from palmitic acid-1-C^{14} is represented in Fig. 1. Within 60 minutes oxidation is completed in systems activated by either glycolic acid or lactic acid. It is noteworthy to point out, however, that the lactic acid system never attains the same level of completion as that of the glycolic acid system. This may be in line with the observation that lactic acid is required in higher concentrations than glycolic acid before it reaches the level of activation attained by the latter acid.

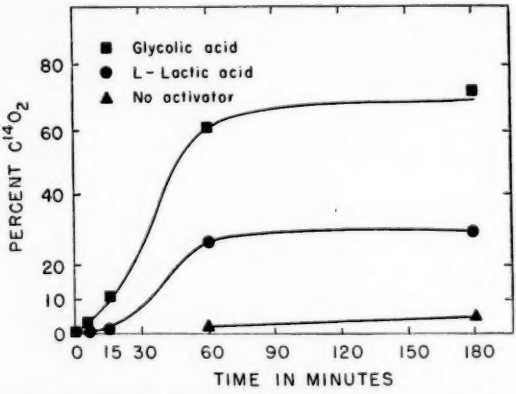

FIG. 1. Effect of reaction time on $C^{14}O_2$ production from oxidation of palmitic acid-1-C^{14} by S_{AS} in the presence of 10 μmoles of activators.

Effect of α-Hydroxy Acid Concentration—As depicted in Fig. 2, glycolic acid activates the system at a concentration level considerably lower than that of lactic acid. Thus at a total concentration of 1.0 μmole the release of $C^{14}O_2$ in 1 hour is about 6.5 times greater with glycolic acid than with lactic acid. It is possible that the affinity of the enzyme (or enzymes) for glycolic acid is greater than that for lactic acid. However, kinetic analysis of the α-hydroxy acid activation is difficult because of a non-linear relationship of reaction rate and enzyme and substrate concentration. It appears that, in part, non-linearity exists because of the adsorptive capacity of the proteins of the S_{AS} fraction for palmitic acid. Thus, although an increase in enzyme concentrations should increase the rate of release of $C^{14}O_2$, a decrease of $C^{14}O_2$ formation is always observed, suggesting that, with an increased protein concentration, more protein becomes available for the adsorption and removal of substrate. In line with this conclusion is the observation that the addition of 10 mg. of serum albumin to the

conventional reaction mixture will completely prevent the formation of $C^{14}O_2$.

Substrate Specificity—Saturated fatty acids labeled in the carboxy group were incubated with S_{AS} under optimal conditions and $C^{14}O_2$ production

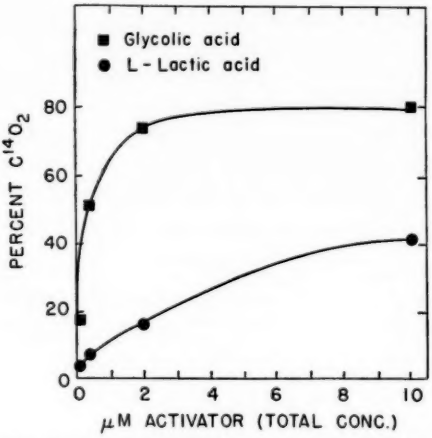

FIG. 2. Effect of activator concentration on $C^{14}O_2$ production from oxidation of palmitic acid-1-C^{14} by S_{AS}.

TABLE III

Substrate Specificity

Substrate	C atom No.	Per cent total $C^{14}O_2$ as $BaC^{14}O_3$
Stearic acid-1-C^{14}	18	42
Palmitic "	16	42
Myristic "	14	32
Lauric acid-1-C^{14}	12	<2
Capric "	10	<1
Caprylic acid-1-C^{14}	8	0

was assayed. Table III indicates that the specificity of the system is rather narrow; fatty acids lower than 14 carbon atoms are inert. No information is available for fatty acids with chain lengths greater than 18 carbon atoms. Palmitic acids-2-C^{14} and -3-C^{14}, glycolic acid-1-C^{14}, acetic acid-1-C^{14}, and butyric acid-1-C^{14} are all inert.

Inhibitor Studies—In order to gain some insight into the mechanism of palmitic acid oxidation, many compounds known to inhibit specific enzyme reactions have been tested. Fluoride and malonate at a final concentra-

tion of 50 μmoles are inert. Iodoacetamide and Cu^{++} at a final concentration of 3 μmoles, DNP at 15 μmoles, and arsenate at 5 μmoles are also inactive.

Effective inhibitors include hydroxylamine, semicarbazide, arsenite, and imidazole. While fairly high concentrations of the first three compounds are required for complete inhibition, imidazole is active at low concentrations, as summarized in Table IV.

Since these four compounds markedly reduce $C^{14}O_2$ production, it was hoped that they were blocking enzymes at various levels of the system and that a search for intermediates, which would presumably be accumulating under these conditions, would reveal the nature of the reaction sequence. However, paper chromatography of the reaction mixtures in the presence

TABLE IV

Inhibitor Studies

Inhibitor	Final concentration	Per cent total $C^{14}O_2$ as $BaC^{14}O_3$
	μmoles	
None		26
Hydroxylamine	0.2	25
	1.0	18
	5.0	5
Arsenite	0.2	18
	1.0	8
	5.0	4
Imidazole	0.2	2
	1.0	1
	5.0	0

or absence of inhibitors with either butanol-formic acid or ethyl acetate-water system did not reveal any new radioactive spot. On analysis reaction mixtures failed to yield any volatile acids such as formic or acetic acid. When hydroxylamine was employed as inhibitor, no evidence could be found to suggest the formation of a hydroxamic acid.

The imidazole effect is highly specific. Imido-4,5-carboxylic acid, urocanic acid, histidine, pyrrole, indole, indoleacetic acid, and benzimidazole were all inert in the test system. Stadtman and White (6) have shown that imidazole will act as an acyl acceptor to form acylimidazole which is rapidly hydrolyzed to the free acid and imidazole. It was hoped that because of the marked inhibitory effect of imidazole in this system, labeled fragments would be accumulated by being channeled away from the path of $C^{14}O_2$ production. However, a search for acidic fragments was unsuccessful. It is possible that imidazole displays its effect in the initial stage of palmitate oxidation if it is assumed that palmitate first forms a palmityl-

enzyme complex. In the presence of imidazole, activated palmityl, instead of proceeding through the normal pathway, is transferred to imidazole to form a labile palmitylimidazole which is then hydrolyzed to regenerate palmitic acid. Thus imidazole would participate in a cyclic, short circuiting mechanism.

Evidence for Additional Activator—After it was established that the system required glycolic acid or one of its analogues presumably as a cosubstrate, it became necessary to investigate the system for other activator requirements.

When S_{AS} is treated with Dowex 1-Cl^- and charcoal (as described under "Experimental"), not only glycolic acid but also boiled S_{AS} had to be added to restore activity, as summarized in Table V. Since the material associated with S_{AS} is readily adsorbed by Dowex 1 and charcoal, the possibility existed that a nucleotide was responsible for the activation. Therefore, DPN, TPN, GDP, ITP, UTP, CTP, ATP, FAD, CoA, and TPP were tested singly and in combination with S_{ASR} for activation effects but with no success. Preliminary tests indicate that the activator associated with S_{AS} is acid-labile, since exposure to 1 N HCl at 100° for 5 minutes destroyed the activator effects of boiled S_{AS}.

TABLE V

Evidence for Additional Activator

See "Experimental" for preparation of S_{ASR}.

Enzyme	Glycolic acid	Boiled S_{AS}	Per cent total $C^{14}O_2$ as $BaC^{14}O_3$
	μmoles	*ml.*	
S_{ASR}	20		3
		0.8	2
	20	0.8	14
S_{AS}	20		41
		0.8	7
	20	0.8	50

Possible Intermediates—If it is assumed that either α- or β-hydroxy or keto derivatives of palmitic acid are intermediates of the oxidation reaction, then the addition of these acids to a system oxidizing palmitic acid should have a marked effect on $C^{14}O_2$ formation. As indicated in Table VI, low concentrations of the α derivatives had a profound inhibitory effect on the formation of $C^{14}O_2$, while the β derivatives were ineffective. This effect may be related to a competitive inhibition by the α derivatives of an essential step in the reaction sequence.

Because of the marked inhibitory effects by α-hydroxy- and α-ketopalmitic acids, α-hydroxypalmitic and α-ketopalmitic acids-1-C^{14} were tested

for activity. As shown in Table VII, while the α-hydroxy derivative was inert, the α-keto acid was decarboxylated more readily than palmitic acid, an essential requirement if the oxidation of α-ketopalmitic acid is part of the reaction sequence for the oxidation of palmitic acid.

TABLE VI

Effect of α-Hydroxy-, α-Keto-, β-Hydroxy-, and β-Ketopalmitic Acids upon Release of $C^{14}O_2$ from Palmitic Acid-1-C^{14} with S_{AS}

Palmitic acid-1-C^{14}	α-Hydroxy-palmitic acid	α-Ketopalmitic acid	β-Hydroxy-palmitic acid	β-Ketopalmitic acid	Per cent total $C^{14}O_2$ as $BaC^{14}O_3$
μmole	*μmole*	*μmole*	*μmole*	*μmole*	
0.1					29
0.1	0.1				3
0.1	0.3				3
0.1		0.1			0
0.1		0.3			0
0.1			0.1		14
0.1					
0.1				0.1	19
0.1				0.3	20

TABLE VII

Comparison of Oxidation of Carboxyl-Labeled Palmitic Acid, α-Hydroxypalmitic Acid, and α-Ketopalmitic Acid

Enzyme system	Palmitic acid	α-Hydroxy-palmitic acid	α-Ketopalmitic acid	Glycolic acid	Per cent total $C^{14}O_2$ as $BaC^{14}O_3$
	μmole	*μmole*	*μmole*	*μmoles*	
S_{AS}	0.1				1
		0.1			1
			0.1		31
	0.1			20	17
		0.1		20	1
			0.1	20	46
Boiled enzyme			0.1	20	0

Furthermore, imidazole and arsenite have no inhibitory effects on the release of $C^{14}O_2$ from α-ketopalmitic acid-1-C^{14}; glycolic and lactic acids have no activating effects. While it is of course obvious that the results of these experiments do not represent conclusive evidence for α-ketopalmitic acid as an intermediate, it is conceivable that this acid is part of the reaction sequence. If so, the site of action of arsenite and imidazole as well as that of glycolic acid precedes the oxidative decarboxylation of α-ketopalmitic acid. The observation that α-hydroxypalmitic acid-1-C^{14} is inert

in the system can best be explained if it is assumed that this acid forms a complex with its specific enzyme and is oxidized to the corresponding keto acid only when held as an enzyme complex. Thus free α-hydroxypalmitic acid would not be in equilibrium with the bound acid and thus would not be oxidized. However, as a condition of oxidative decarboxylation, the α-ketopalmitic acid would be released from the enzyme complex in order to participate in the decarboxylation reaction.

Future investigations designed to isolate intermediates of palmitic acid-1-C^{14} oxidation with non-radioactive α-ketopalmitic acid as carrier will provide the necessary unequivocal evidence for this postulate.

SUMMARY

Soluble extracts prepared from cotyledons of germinating seeds catalyze the oxidation of palmitic acid-1-C^{14}, the carboxyl carbon being converted to carbon dioxide. No radioactive carbon is recovered in the respiratory carbon dioxide when palmitic acid labeled in positions other than the carboxyl group is incubated with these soluble extracts.

The reaction is stimulated by heat-stable soluble substances present in the extracts. These substances were identified as glycolic and lactic acids. No requirement for either ATP, CoA, cocarboxylase, Mg^{++}, Cu^{++}, or Krebs cycle intermediates could be demonstrated. The reaction is inhibited by imidazole, hydroxylamine, and sodium arsenite. Iodoacetamide, fluoride, malonate, azide, copper, dinitrophenol, and arsenate do not inhibit the system.

The system is equally active with labeled stearic, palmitic, and myristic acids, weakly active with lauric acid, and inert with capric and lower acids. The effect of the following variables has been studied: reaction time, pH, activator (glycolic or L-lactic acid) concentration, and substrate concentration.

Carboxyl-labeled 2-hydroxypalmitic acid and 2-ketopalmitic acid were synthesized. The former was found to be inert in the reaction systems, but the latter is rapidly attacked with release of the label in the respiratory carbon dioxide.

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THE AMINO ACID REQUIREMENTS OF MAN

X. THE LYSINE REQUIREMENT*

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Two of the earlier papers of this series dealt with the minimal requirements of adult man for tryptophan (1) and phenylalanine (2), as measured by the maintenance of nitrogen equilibrium. In continuation of this program, attention was next directed to the lysine needs of the human organism. This amino acid is of special interest since many vegetable proteins have a low content or are practically devoid of it. Particularly is this true of certain cereal proteins which serve as important constituents of human dietaries. Hence, the question of the minimal lysine requirement of man assumes unusual significance. The experiments outlined in the following pages demonstrate that surprisingly small quantities of this amino acid suffice, at least for the maintenance of nitrogen equilibrium in adults.

EXPERIMENTAL

Elsewhere, the reader will find detailed descriptions of the methods used in the conduct of the experiments (3, 1). In the present investigation, the customary procedures were followed except with respect to the composition of the amino acid mixtures. Throughout the entire program of human studies, the make-up of these mixtures has been modified progressively, as experience warranted, in order thereby to conserve supplies of costly materials.

Two lysine experiments are presented in detail below. To save space, the actual data obtained in four additional tests are omitted, but the fig-

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† The experimental data in this paper are taken from theses submitted by Aleck Borman and Minor J. Coon in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois. Present address of Aleck Borman, The Squibb Institute for Medical Research, New Brunswick, New Jersey. Present address of Minor J. Coon, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

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ures representing the minimal requirements of the subjects will be revealed later in this paper.

The first subject, R. L. B., received initially a daily diet consisting of amino acid Mixture 87 (Table I), Wafers IV (the composition of which has been described in a previous paper (4)), 125.5 gm. of additional sucrose, and 13.65 gm. of additional butter fat. The second subject, C. H. E., received initially a daily diet composed of amino acid Mixture 136 (Table I), Wafers IV, 202.2 gm. of additional sucrose, and 25.90 gm. of additional butter fat. Cellu flour, to provide a residue in the alimentary tract, and the vitamin pills (*cf.* (3)) were administered apart from the basal rations. The daily allotment of Mixture 87 furnished 9.77 gm. of nitrogen, of which 1.20 gm. were derived from the D isomers of valine, isoleucine, and threonine. The daily allotment of Mixture 136 furnished 9.79 gm. of nitrogen, of which 1.07 gm. were derived from the D isomers of valine, leucine, isoleucine, and threonine. From all sources, the daily nitrogen intakes of Subjects R. L. B. and C. H. E. amounted to 10.08 and 10.10 gm., respectively, of which 0.31 gm. in each instance was of an unknown nature. This had its origin largely in the starch present in the wafers and in lesser quan-

TABLE I

Composition and Daily Intakes of Amino Acid Mixtures

Component	Mixture 87		Mixture 136	
	As used	N content	As used	N content
	gm.	gm.	gm.	gm.
Valine	8.56*	1.02	2.40*	0.29
Leucine	4.91	0.52	9.82*	1.05
Isoleucine	6.16*	0.66	6.16*	0.66
Methionine	3.68*	0.35	3.68*	0.35
Threonine	6.16*	0.72	1.20*	0.14
Phenylalanine	3.00	0.25	3.00*	0.25
Tryptophan	0.20	0.03	0.25	0.03
Lysine monohydrochloride	1.24†	0.19	1.50†	0.23
Glycine	21.60	4.03	25.68	4.79
Urea	4.29	2.00	4.29	2.00
	59.80	9.77‡	57.98	9.79§

* Racemic acids.

† Equivalent to 1.00 and 1.20 gm. of free lysine, respectively.

‡ Of the nitrogen contributed by this mixture, 1.20 gm. were derived from the D isomers of valine, isoleucine, and threonine.

§ Of the nitrogen contributed by this mixture, 1.07 gm. were derived from the D isomers of valine, leucine, isoleucine, and threonine.

tities in the butter fat, liver concentrate, and lemon juice (*cf.* (3)). Each ration furnished 55 calories per kilo of initial body weight.

The results of the experiments upon Subjects R. L. B. and C. H. E. are summarized in Table II. The lysine dosages are expressed in terms of the free amino acid, although the compound was administered in the form of

TABLE II

Lysine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
Subject R. L. B.						
6	63.1	10.08	8.27	0.53	+1.28	1.0 gm. L-lysine†
5	64.0	10.08	10.31	0.77	−1.00	No L-lysine
9	64.0	10.08	9.20	0.72	+0.16	0.4 gm. L-lysine
7	64.9	10.08	9.43	0.68	−0.03	0.3 " "
7	64.9	10.08	9.63	0.65	−0.20	0.2 " "
4	64.4	10.08	10.40	0.63	−0.95	0.4 " D-lysine
Subject C. H. E.						
7	69.4	10.10	8.52	0.98	+0.60	1.2 gm. L-lysine†
4	70.3	10.10	9.74	0.87	−0.51	0.6 " "
7	70.8	10.10	9.00	0.72	+0.38	0.8 " "
6	71.2	10.10	9.00	0.76	+0.34	0.7 " "

* The initial diets of Subjects R. L. B. and C. H. E. contained amino acid Mixtures 87 and 136, respectively.

† Throughout both experiments the lysine was administered as the monohydrochloride, but for the convenience of the reader the daily dosage is expressed as the free amino acid.

the monohydrochloride. During a single period, D-lysine replaced the L isomer and was ingested as the dihydrochloride. Sufficient sodium bicarbonate to neutralize the hydrochloric acid present in the amino acid salts was always incorporated in the rations. In each experiment, as alterations in the lysine intake were made, the nitrogen content of the food was maintained at a constant level by appropriate adjustments in the amount of glycine present in the amino acid mixture.[1]

[1] As stated repeatedly in papers of this series, glycine and urea were incorporated in the amino acid mixtures to furnish nitrogen for the synthesis of the non-essential amino acids.

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The data in Table II show that Subject R. L. B. attained a strongly positive balance with a daily intake of 1.0 gm. of L-lysine. During the second period, for a reason which will be disclosed later, lysine was excluded from the diet. As was to be anticipated, the subject immediately experienced a negative balance and showed an average daily loss of 1.00 gm. of nitrogen. Thereafter, positive nitrogen balance resulted from the daily consumption of 0.4 gm. of L-lysine but not from dosages of 0.3 and 0.2 gm. per day. Obviously, the minimal daily requirement of this subject was 0.4 gm. During the final period of the test, the ingestion of 0.4 gm. of the D isomer daily induced a negative nitrogen balance of approximately the magnitude observed when the ration was devoid of lysine. Like results followed the administration to another subject (A. B.) of 0.6 gm. of D-lysine. This was 50 per cent in excess of his minimal L-lysine requirement.[2] On the basis of these observations, one may affirm with considerable confidence that little, if any, of the D isomer can be utilized by adult man.

The experiment upon Subject C. H. E. (Table II) was conducted a year after that upon Subject R. L. B. In the meantime, information acquired in other investigations warranted marked reductions in the valine and threonine content of the basal diets. These changes are reflected in the comparative composition of amino acid Mixtures 87 and 136 (Table I). *Parenthetically*, it is worthy of note that, of the 9.79 gm. of nitrogen furnished by the daily allotment of Mixture 136, 6.79 gm. were derived from glycine and urea. An additional 1.07 gm. of nitrogen originated in the D isomers of valine, leucine, isoleucine, and threonine, and may not have been available for the uses of the organism. Thus, if the incomplete utilization of D-phenylalanine (*cf.* (2)) is disregarded, the nitrogen derived from the physiologically active amino acids in Mixture 136 was 1.93 gm. It is little short of astounding that this amount of nitrogen in the form of an appropriate mixture of L-amino acids is sufficient to meet the daily needs of man, provided that the mixture also furnishes abundant supplies of glycine and urea as sources of nitrogen for the synthesis of the nonessential amino acids. Yet, many experiments in this and subsequent papers will prove that nitrogen equilibrium can be maintained under these circumstances. One cannot escape the conviction that mixtures of the essential amino acids, *when the amounts of the latter are adjusted to the approximate needs of the human subject for them*, are remarkably efficient sources of nitrogen. This aspect of the problem will be discussed in greater detail at a later date when the minimal requirements of man for the other essentials have been considered.

[2] The details of this test are omitted, since the findings agreed completely with those obtained in the experiment upon Subject R. L. B.

To return to the question of the lysine requirement, the data in Table II show that Subject C. H. E. attained a strongly positive nitrogen balance on a diet carrying amino acid Mixture 136. This supplied 1.2 gm. of L-lysine daily. Reducing the lysine intake to 0.6 gm. daily was followed by a negative nitrogen balance, during which the average daily loss of this element amounted to 0.51 gm. In the two succeeding periods, the lysine consumption was first raised to 0.8 gm. and then lowered to 0.7 gm. daily. At each of these levels the subject manifested a positive nitrogen balance. Clearly, the minimal daily L-lysine requirement of C. H. E. was 0.7 gm.

Table III lists the findings in six lysine experiments. Unfortunately, the test upon Subject R. J. D. was incomplete. Several months earlier,

TABLE III

Summary of L-Lysine Experiments

Subject	Body weight	Minimal daily requirement*
	kg.	*gm.*
R. L. B.	63.1	0.4
G. E. M.	65.8	0.7
R. J. D.	67.1	0.6 or less†
C. H. E.	69.4	0.7
A. B.	69.9	0.4
H. D. P.	71.7	0.8

* Throughout all experiments the lysine was administered as the monohydrochloride, but the requirements are here expressed as the free amino acid.

† It became necessary to discontinue this experiment before the exact minimal requirement of the subject had been established; however, 0.6 gm. per day was an adequate intake.

this young man had served as a subject in one of the tryptophan experiments (1). The end of the spring semester rendered it impracticable for him to continue on the lysine test for a sufficient length of time to permit the establishment of his exact minimal requirement. With a daily intake of 0.6 gm. he was in positive balance; consequently, his minimal needs could not have exceeded this figure.

The subjects listed in Table III did not vary widely in size; however, the data show clearly that no observable correlation exists between the body weight of an individual and his lysine requirement. Furthermore, no relationship was detected between the lysine requirement and the body surface or creatinine output. Like conclusions were reached in the tryptophan (1) and phenylalanine (2) tests. On the other hand, relatively wide differences (0.4 to 0.8 gm.) were observed in the minimal lysine requirements of different individuals. In this respect, lysine proved to be one of the most variable of the entire group of essential amino acids.

In accordance with the practice followed with respect to tryptophan and phenylalanine, and for the reasons set forth elsewhere (1), the highest value observed in the six lysine experiments, or 0.8 gm., is proposed *tentatively* as the minimal daily requirement of adult man. One cannot exclude the possibility that individuals other than those tested may have lysine requirements in excess of the above figure. A statistical analysis of the data in Table III, with the assumption that 0.6 gm. actually represented the minimal needs of Subject R. J. D., reveals the fact that the odds are overwhelmingly against the probability that any adult male would have a requirement equal to or greater than twice the proposed minimal level, or 1.6 gm. of L-lysine daily. Therefore, one may confidently affirm that 1.6 gm. of this amino acid is a *safe* daily intake for an adult. Experience with a much larger number of individuals may eventually warrant a decrease in this figure. In the course of our investigations, thirty-four different subjects have been kept in positive nitrogen balance upon daily intakes of 1.6 gm. or less of L-lysine, and no individual has been found who could not be so maintained.[3]

The surprisingly small quantities of lysine which are necessary for nitrogen balance are of peculiar interest and significance from the nutritional point of view. The low content of this amino acid in certain vegetable proteins has led some investigators to express the fear that lysine may be a limiting factor in human dietaries. Indeed, the suggestion is sometimes heard that bread should be enriched with lysine in order to safeguard the public against an inadequate intake. Admittedly, the ultimate goal of nutritional biochemistry is not merely to prevent deficiencies but to insure *optimal* intakes of essential nutrients. It is true also that the experiments herein described do not answer the question: What is the *best* daily intake of lysine for adult man? On the other hand, it seems reasonable to suppose that, for any dietary component, the smaller the minimal requirement of the organism for it the smaller will the optimal intake prove to be, if and when techniques are devised which enable one to measure this quantity. At the moment, the most that can be done is to determine the minimal essential needs of man and then to furnish what appears to be a reasonable excess. Fortunately, estimates of the consumption of amino acids by different economic groups in this country are available. Using figures for food purchases collected during the spring of 1942 by the United States Department of Agriculture, Block and Bolling (5) have calculated

[3] Of the thirty-four subjects mentioned above, four, in addition to those listed in Table III, were maintained in nitrogen balance upon daily intakes of 0.8 gm. or less, but no effort was made to establish their exact requirements. Thus, 0.8 gm. of L-lysine daily was sufficient for ten individuals at least. In later studies involving other amino acid requirements larger quantities of lysine were administered as a precautionary measure, although 0.8 gm. might have been adequate for the purpose.

the average daily per capita intake of each essential amino acid. The results show that the lowest income group of urban families consumed approximately 4 gm. each of lysine per day. In all probability the survey figures for food consumption were too high, since those who assembled them found no practical way of making allowance for household waste. If one assumes that wastage amounted to 25 per cent, a discount which would seem to be sufficiently generous, the average daily lysine intake would still be almost twice the *safe* level for an adult. Whether the growing child requires more than this remains to be determined. Furthermore, in certain areas of the world, where bread constitutes a much larger proportion of the total food of the adult than in the United States and where little or no animal protein is consumed, some method of artificially raising the lysine content of the diets may prove to be advantageous.

TABLE IV

Relation of Lysine to Output of Organic Acids

Period averages.

Subject	Period	Average daily output of organic acids	Daily intake of lysine*
	days	*ml. of 0.1 N*	
R. L. B.	6†	542	1.0 gm. L-lysine
	5	536	No L-lysine
	4†	537	0.4 gm. L-lysine
A. B.	7†	654	0.4 " "
	9	650	0.3 " "
	5	663	No L-lysine
	8†	685	0.4 gm. L-lysine
	4	678	0.6 " D-lysine

* Expressed as the free amino acid.

† Subjects were in positive nitrogen balance during these periods only.

One additional point regarding lysine requires brief consideration at this time. More than a decade ago, Albanese *et al.* (6) reported that human subjects receiving diets deficient in this amino acid not only complain of "nausea, dizziness and hypersensitivity to metallic sounds," but also manifest a sharp rise in the urinary output of non-ketonic organic acids as measured by the procedure of Van Slyke and Palmer (7). In our earlier experiments with lysine-deficient rations (8), no *unique* subjective symptoms were encountered. The young men experienced a loss of appetite, an increase in nervous irritability, and a sensation of fatigue, such as are observed following the exclusion from the food of any one of the essential amino acids. However, the urines were not examined for organic acids.

Inasmuch as changes, if they occur, in the excretion of these acids might be of distinct value in the detection of lysine deficiencies, the current experiments were used as an opportunity of reinvestigating this phenomenon. Consequently, lysine was removed from the diet of Subject R. L. B. (Table II) for the duration of the second period, and the output of organic acids was determined daily throughout the first and second periods and the first 4 days of the third period. Similarly, in the experiment upon Subject A. B. (Table III), the details of which are omitted from this paper, the output of organic acids was measured for 33 days. The results obtained in both tests are presented in summary form in Table IV. As will be observed, no significant alteration in the excretion of organic acids followed the exclusion of lysine or changes in the amount present in the food. Perhaps the difference between our findings and those of Albanese and his associates is to be accounted for by the fact that the latter investigators used casein which had been deaminized and then hydrolyzed as the source of nitrogen in their lysine-free rations. This material induces an anemia in rats (9) of the macrocytic megaloblastic type, due to the presence of a toxic agent (10).

SUMMARY

Six experiments have been conducted upon normal young men for the purpose of establishing their minimal L-lysine requirements, as measured by the nitrogen balance technique. The results show that the subjects required from 0.4 to 0.8 gm. of this amino acid per day. No correlation was observed between the minimal needs of the subjects and their body weights, body surfaces, or creatinine output. In accordance with the practice followed in similar investigations involving other amino acids, it is proposed, as a precautionary measure, that the highest observed value, or 0.8 gm., be designated *tentatively* as the minimal daily L-lysine requirement of man.

In formulating human diets designed to avoid amino acid deficiencies, allowance should be made for the possibility that individuals other than those tested may require still larger intakes of L-lysine. For this purpose, twice the highest observed minimum, or 1.6 gm. daily, is certainly a *safe* intake. Eventually, experience may permit this quantity to be lowered.

In two subjects, the administration of D-lysine, in amounts equal to or greater than their L-lysine requirements, immediately induced negative nitrogen balances. The findings demonstrate that little, if any, of the D isomer is utilized by man.

No evidence was obtained in support of the alleged increase in output of organic acids following the exclusion of lysine from the food.

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THE REACTION OF METHYL MERCURY NITRATE WITH THE SULFHYDRYL GROUPS OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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The importance of two of the thiol groups of yeast triosephosphate dehydrogenase for its activity has been shown by the abolition of activity and diphosphopyridine nucleotide binding by the addition of 2 equivalents of *p*-chloromercuribenzoate (PCMB) (1, 2). The reaction of methyl mercury nitrate with this enzyme has been found to be anomalous in the sense that this mercury compound, unlike PCMB, does not react first with the enzyme sulfhydryl groups which are required for activity. These results and some determinations of the total numbers of sulfhydryl groups available in the enzyme after various treatments are presented here.

EXPERIMENTAL

The enzyme was prepared from Fleischmann's yeast by the procedure of Krebs *et al.* (3). It has not been found possible to obtain preparations fully active in the absence of cysteine, even if ethylenediaminetetraacetic acid (EDTA) is present during all stages of the preparation. It has also been found that the enzyme loses activity on storage (whether activity is tested with or without cysteine) even in the presence of EDTA. In general, the enzyme has 20 to 30 per cent less activity with EDTA than after cysteine activation. Enzyme concentrations were determined by absorption at 280 mμ and corrected for any nucleic acid by the absorption at 260 mμ (3, 4). The enzyme was dialyzed against a phosphate buffer (pH 6.7 or 7.9) before use.

The diphosphopyridine nucleotide (DPN) used was a Pabst Laboratories preparation and assayed 90 per cent pure by reduction with excess substrate.

DL-Glyceraldehyde-1-bromide-3-phosphoric acid dimer was kindly furnished as the dioxane addition compound by Dr. C. A. Dekker and assayed 95 to 100 per cent pure.

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The methyl mercury bromide used was generously supplied by Dr. P. E. Wilcox. The PCMB used was a Sigma preparation.

Enzyme activity was determined in the following system. 2.6 ml. pyrophosphate buffer (0.03 M) at pH 8.3 containing 0.0024 M EDTA, 0.1 ml. of enzyme solution (usually containing 0.001 mg. of protein), and 0.1 ml. of 0.195 M sodium arsenate were mixed. The reaction was started by the addition of 0.2 ml. of a solution containing 0.15 mg. of the triose phosphate addition compound and 1.6 mg. of DPN. The optical density at 340 mμ was read at 0.5 minute intervals against a blank containing buffer and the triose phosphate-DPN mixture. The final pH was 8.1 to 8.3. Activities were calculated as a first order rate constant from the 3.0 minute reading. With fresh cysteine-activated preparations k_1 is usually 180 to 200 ml. per minute per mg. of protein at this level of triose phosphate. The reaction follows no simple kinetics exactly, but approximates a first order reaction, particularly if the 0.5 minute reading, which is generally high, is neglected. In some cases the reaction was started by the addition of triose phosphate alone, the DPN having been put in the reaction mixture previously. In this case the activity is 10 to 20 per cent higher than that observed with the other method of starting the reaction. All reactions were performed in duplicate.

PCMB concentrations were determined by direct titration with dithizone, standardized against mercuric nitrate, as for the determination of methyl mercury (5). Sulfhydryl groups in the native protein were determined by dithizone titration of the unchanged methyl mercury extracted from the aqueous protein solution into a toluene layer.[1] Equilibration of the two phases was carried out in a rocking dialyzer at 0–2°. Sulfhydryl groups in the denatured protein were determined by direct titration with methyl mercury (5). All treatments of the protein with mercurials were carried out in phosphate buffers, pH 6.7 or 7.9.

Results

Table I contains the results of the determinations of sulfhydryl groups in the native protein and in the guanidine hydrobromide-denatured (5) protein. The number of sulfhydryl groups found on the native molecule, namely four, is similar to the number of sulfhydryl groups which react with iodoacetic acid in the case of triosephosphate dehydrogenase from muscle (6). The total number of sulfhydryls (six and a half after denaturation) is, however, considerably less than in the latter protein (7). The figures for the sulfhydryl groups of the native and denatured protein are likely to be lower than the true values; *i.e.*, the values for a protein preparation which is fully reduced. If it is considered that 20 per cent of the protein

[1] Unpublished method of Dr. W. L. Hughes, Jr., transmitted by Dr. P. E. Wilcox.

sulfhydryl groups are oxidized (judging from a 20 per cent activation by cysteine which was observed with the preparations used for these determinations), the figures become 5.0 and 8.1 for sulfhydryl groups of the native and denatured protein.

It appears from Fig. 1 that methyl mercury reacts first, to a large extent at least, with the two sulfhydryl groups which are not primarily involved (1, 2) in the activity of the enzyme. This is indicated by the 74 per cent of activity remaining after the addition of 2 equivalents of methyl mercury as against the 50 per cent to be expected from random reaction with four sulfhydryl groups per mole of protein. The presence of some oxidized, or

TABLE I

Sulfhydryl Groups of Yeast Glyceraldehyde-3-phosphate Dehydrogenase

Preparation No.	Age of preparation	Activity,* k_1, ml. per min. per mg. protein	Treatment before sulfhydryl determination	Sulfhydryl groups per 120,000 gm.
	mos.			
K21-3	1	145	None	4.0
K21-3	1		Denatured with guanidine HBr†	6.5
K32-2	6	117	None	3.6
04-5‡	1	113	"	4.0
04-5	1.5		4 equivalents PCMB, reprecipitated with $(NH_4)_2SO_4$	2.1

* Without cysteine activation.

† Edelhoch *et al.* (5).

‡ This preparation was activated with cysteine, recrystallized two times, and stored with 0.005 M EDTA. The activity was initially $k_1 = 170$.

otherwise inactive enzyme, as is known to occur with urease (8), does not affect this conclusion. The per cent inhibition after reaction with a mercurial equivalent in amount to a given fraction of the total native sulfhydryl groups present is predictable on the basis of random reaction regardless of the fraction of the total native sulfhydryl groups required for activity. PCMB appears to react only with the sulfhydryls required for activity (Table I and Fig. 1). The two mercurials are different both in the size of the organic part and in that one (PCMB) bears a charge other than that on the mercury. Either or both of these differences may be relevant to the observed difference in reactivity. It may be noted that, although there are four sulfhydryl groups reactive to methyl mercury, 4 equivalents of this reagent do not completely abolish the activity of the enzyme (Fig. 1). A similar situation obtains with PCMB. A lack of good agreement has been observed in duplicates of assays of activity when the inhibition is nearly

complete, which appears to indicate some reactivation of the enzyme in the test system.

The experiments of Fig. 1 show that the four sulfhydryls of the enzyme molecule are of two kinds with respect to their reactivity with methyl mercury as well as with respect to their necessity for enzymatic activity. Since about 25 per cent of the enzymatic activity (instead of none) is lost by the addition of 2 equivalents of methyl mercury, the interpretation that only two sulfhydryls are necessary for activity and that methyl mercury reacts solely with the two sulfhydryls not necessary for activity is inadequate. We may suppose that either the reaction of methyl mercury is not completely specific between the two classes or the two unnecessary sulfhydryls do have some effect on the activity of the molecule. An experiment designed to test whether the reaction of methyl mercury with the protein was completely selective for the two unessential sulfhydryl groups was inconclusive. The protein was first treated with 2 equivalents of methyl mercury, then with 2 equivalents of PCMB, and, after reprecipitation with ammonium sulfate, the residual sulfhydryl was found to be 0.52 equivalent per mole. This would indicate an incomplete specificity of the methyl mercury reagent, assuming that PCMB reacts only with the two essential sulfhydryl groups (Table I). However, a similar experi-

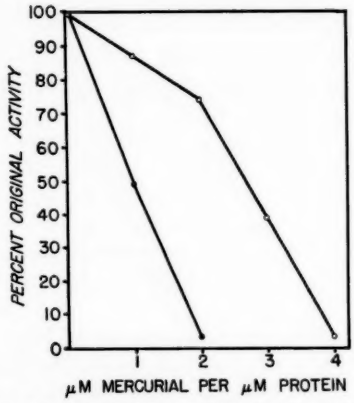

FIG. 1. Per cent loss of activity with the addition of methyl mercury nitrate (○) or *p*-chloromercuribenzoate (●) to the enzyme in 0.1 M phosphate buffer, pH 6.7 or 7.9. The points for 1 and 2 equivalents of methyl mercury are averages of five determinations in duplicate; the other points are averages of two determinations in duplicate. The percentages are not corrected for the presence of inactive enzyme in the preparations used (see the text). The preparations contained four sulfhydryl groups per native molecule.

ment in which the protein was treated with 4 equivalents of methyl mercury gave a residual sulfhydryl only of 0.39 equivalent per mole. These results are probably caused by denaturation of the protein, as seems likely from the rapid appearance of protein precipitate in both experiments during the equilibration with toluene. Considering the amount of preliminary handling and the high concentration of protein (which has been observed by us to enhance denaturation of this protein) necessary for these determinations, it seems not unlikely that sufficient denaturation to account for the results may have occurred. In these circumstances the closeness of the figures for residual sulfhydryl in the two experiments may indicate that the methyl mercury reagent reacts completely with the unessential sulfhydryl groups before reacting with those necessary for enzymatic activity. If this is so, it becomes necessary to suppose that these groups do have a small effect on the activity of the enzyme.

SUMMARY

The number of sulfhydryl groups in the yeast triosephosphate dehydrogenase molecule has been determined for the native and guanidine hydrobromide-denatured enzyme by the use of methyl mercury.

The reaction of methyl mercury nitrate with yeast triosephosphate dehydrogenase indicates that the most reactive of the four sulfhydryl groups of the native molecule are the two not required for enzymatic activity.

The author wishes to thank Dr. Hans Neurath, in whose laboratory this work was performed, Dr. P. E. Wilcox for furnishing information on the use of methyl mercury, and Dr. E. G. Krebs, who supplied some of the enzyme samples.

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SOME ASPECTS OF THE METABOLISM OF LECITHIN AND ITS DERIVATIVES IN LIVER*

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Recent evidence has renewed speculation as to a possible rôle of phospholipides in the oxidation of fatty acids. Artom (1) has proposed that a choline-containing compound, which is preformed in the tissues *in vivo*, is necessary for the oxidation of fatty acids in isolated systems. Greenbaum and McClean (2) have noted a remarkable agreement between the fatty acid oxidase capacity and the changes in neutral fat content of the liver of animals during treatment with growth hormone. On the basis of other observations (3), they suggest that neutral fat is first converted into phospholipide, which is, in turn, a vehicle for the oxidation of fatty acids.

In a preliminary communication from this Laboratory (4), it was reported that certain lecithin derivatives stimulated oxygen uptake in liver mitochondria. This present paper is a detailed report on these observations.

EXPERIMENTAL[1]

Preparation of Rat Liver—Male rats (Wistar) weighing 150 to 200 gm. were used throughout the experiments and were fed *ad libitum* on a standard chow diet. The animals were fasted for 24 hours prior to the experiments, sacrificed by a sharp blow on the head, decapitated, and the liver rapidly removed and placed on ice. The chilled liver was homogenized in 2 volumes of cold 8.5 per cent sucrose with a Virtis[2] homogenizer operating at top speed for 1 minute. The subcellular fractions were then isolated from this homogenate essentially by the procedure of Hogeboom, Schneider, and Palade (5). All operations were carried out at 0–3°.

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[1] The following abbreviations are used: GPC = glycerylphosphorylcholine; GP = glycerophosphate; PC = phosphorylcholine; CoA = coenzyme A.

[2] Virtis Company, 160 Ashburton Avenue, Yonkers, New York.

XUM

The preparation used for the rat liver studies consisted of 0.5 ml. (15 to 20 mg. dry weight) of a 5 ml. sucrose suspension of mitochondria and 1.0 ml. of the original supernatant fraction.

Preparation of Guinea Pig Liver—Male guinea pigs weighing 250 to 350 gm. were used. They were maintained on a stock chow diet. The animals were stunned by a sharp blow on the head and exsanguinated from the neck vessels. The liver was removed quickly and homogenized for 30 seconds in 3 volumes of 8.5 per cent sucrose in a loose fitting Potter-Elvehjem homogenizer. Subcellular fractions were then isolated from this homogenate essentially by the procedure of Hogeboom, Schneider, and Palade (5). All operations were carried out at 0–5°.

The preparation used for the guinea pig studies consisted of 1.5 ml. (45 to 60 mg. dry weight) of a 25 ml. sucrose suspension of mitochondria derived from 1.0 to 1.5 gm. of liver tissue.

Medium for Manometric Studies—The medium used for the manometric studies on both rat and guinea pig liver enzymes was slightly modified from that of Pardee and Potter (6) and contained the following components at the final concentrations indicated: 0.017 M potassium phosphate buffer (pH 7.3), 0.015 M potassium citrate, 0.0015 M potassium malate, 0.0015 M potassium α-ketoglutarate, 3×10^{-4} M cytochrome *c*, 0.0016 M sodium adenosine triphosphate, 0.06 M sucrose, 0.0033 M $MgCl_2$, enzyme (see above), and water to make a final volume of 3.0 ml. The substrates and enzymes were used in concentrations indicated in the results.

Methods—Oxygen uptake was measured in Warburg vessels by the standard procedures. The rate of shaking the manometers was 110 complete cycles per minute, and all measurements were made in the presence of oxygen at 37°; the CO_2 was collected in the usual manner by use of KOH-treated filter paper in the center well. A 5 to 8 minute temperature equilibration period preceded the start of the manometric measurements.

The controls for the various experiments contained mitochondria in the "sparker" medium described above and, where indicated, in addition the supernatant fraction. The usual oxygen uptake values for the controls were of the order of 750 μl. per hour, and triplicate determinations agreed within 3 to 5 per cent. Although the net oxygen uptake values noted in a particular experiment were low compared to the total oxygen uptake, these values were always consistent and agreed within 3 to 5 per cent. Whereas the values reported in Tables I to IV and Figs. 1 to 5 represent a particular experiment, in every case at least six separate experiments were performed and each showed the same typical results as to the influence of a particular compound on the net oxygen uptake.

Acetoacetate production was determined by the method of Edson (7).

Phosphorus determinations were made by the colorimetric method of King (8).

Radioactive C^{14} was measured with conventional flow counters, and all samples were counted either as infinitely thin layers (direct plating) or as $BaCO_3$, when a standard curve was used to correct for mass absorption.

Substrates—Palmitic acid-1-C^{14} was obtained from the Research Specialties Company[3] and contained 0.1 mc. of C^{14} per mmole (as measured on our counting equipment). A stock solution of the labeled fatty acid in water was prepared by adding 10 mg. to 3.0 ml. of water, followed by the addition of 1 drop of concentrated NH_3. The mixture was made soluble by warming on a steam bath. Excess NH_3 was removed by passing nitrogen into the heated solution. The homogeneity of the fatty acid preparation was tested by counting an aliquot, and it was found that the radioactivity obtained agreed to within 1 per cent of the calculated value. Prior to the addition of the fatty acid to the Warburg cups, the solution was warmed to 60° to effect solution and then allowed to cool to 40°. Since there was no inhibition of the oxygen uptake from the control, and inasmuch as triplicate determinations of the CO_2 formed during any one experiment showed a difference of only 3 to 5 per cent, the methods of preparation and addition of the fatty acid to the cups appeared satisfactory.

Tripalmitin-1-C^{14} was obtained from the Nuclear Instrument and Chemical Corporation and contained 2.54 mc. per mmole (as measured on our equipment). An aqueous suspension was prepared by the addition of 5.8 mg. of the tripalmitin to 5 ml. of water, and the suspension was homogenized in a Potter-Elvehjem glass homogenizer until a very fine emulsion had been obtained. The emulsion was heated to 60° and, prior to addition to the Warburg flasks, was allowed to cool to 40°.

(Dipalmitoleyl)-L-α-lecithin (yeast lecithin) was obtained by a chromatographic procedure (9). The saturated analogue, (dipalmitoyl)-L-α-lecithin, was prepared by the hydrogenation of the above compound (9). β-(Monopalmitoyl)glycerophosphorylcholine (lysolecithin) was prepared by an enzymatic procedure (10). Calcium phosphorylcholine was synthesized by the method of Baer (11). The calcium was removed by an ion exchange resin (IR-100, potassium cycle). In some early experiments, the phosphorylcholine was freed of calcium by reaction with oxalic acid. However, apparently a trace amount of calcium always remained, and it was later found that in concentration as low as 7×10^{-5} M calcium had an inhibitory effect on the stimulatory influence of phosphorylcholine on mitochondrial respiration. Choline was a commercial product and was recrystallized from absolute ethanol. Glycerophosphate was a mixture of the α and β isomers, 53 per cent of which was the α isomer according to

[3] 1148 Walnut Street, Berkeley, California.

periodate titration. Glycerylphosphorylcholine was synthesized by the procedure of Baer and Kates (12).

RESULTS AND DISCUSSION

Oxidation Studies with Rat Liver System. Lecithin—Various systems were studied in order to find the proper conditions for the oxidation of lecithin in the rat liver. In the initial experiments, the fatty acid oxidase system of Kennedy and Lehninger (13), which contained 0.0005 M malate or succinate as "sparkers," was used. However, it was found that this system did not cause any apparent oxidation of lecithin, and, in fact, there was strong inhibition of the oxygen uptake, compared with the controls, when 0.8 μmole or more of lecithin was added. Although no significant net O_2 uptake occurred in the presence of this lipide at low concentration ($<3.5 \times 10^{-5}$ M), there was no inhibition of the oxidative capacity of the enzyme preparations. (Dipalmitoleyl)-L-α-lecithin and (dipalmitoyl)-L-α-lecithin and a purified rat liver lecithin also apparently were not oxidized. Various added cofactors, such as coenzyme A, pyridoxal phosphate, biotin, thiamine pyrophosphate, and folic acid, failed to cause any oxidation of these lecithins.

Pardee and Potter (6) have described a medium which will maintain oxidative phosphorylation from 3 to 4 hours at a constant rate. The principal difference between this medium and that used by Kennedy and Lehninger was in the number of "sparkers" (malate, citrate, and α-ketoglutarate as contrasted to malate alone) and in their concentration (3 to 9 times the concentrations used by Lehninger). In the presence of the medium of Pardee and Potter and with low concentrations of lecithin (0.1 to 0.4 μmole), there was a net oxygen uptake by the mitochondria which was in excess of that required for complete oxidation of the fatty acids of lecithin. However, this does not mean that lecithin was being oxidized in this system, but that it at least stimulated oxygen uptake by a route as yet unknown. In order to obtain consistent net oxygen uptake values, it was found necessary to use a concentrated suspension of rat liver mitochondria fortified with the supernatant fractions indicated in the experimental section. The mitochondria alone were without effect. The time-course for the oxygen uptake values in the presence of unsaturated and saturated lecithins is shown in Fig. 1.

It was found that the influence of lecithin on the net oxygen uptake decreased with an increase in concentration of this lipide and finally became negligible at high concentrations (0.8 μM). The concentration for maximal net oxygen uptake was approximately 0.1 μM. Although the results were variable from one experiment to the next, in a range from 119

to 318 per cent of theory for complete oxidation of the compounds,[4] the results within an experiment agreed within 3 to 5 per cent. There was consistently a higher net oxygen uptake than could be explained by a simple oxidation of the fatty acids of lecithin to CO_2 and H_2O.

Lysolecithin—Inasmuch as lysolecithin is a surface-active compound, it was not unexpected that it, like lecithin, inhibited oxidation when used even in relatively low concentrations (1.0 μmole). On the other hand, it was found that the mitochondria could tolerate higher concentrations of lysolecithin (0.9 μmole) than of lecithin and the concentration for maxima

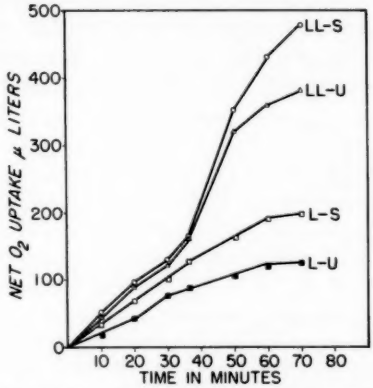

FIG. 1. Effect of lecithins and lysolecithins on net oxygen uptake in rat liver mitochondrial systems. *L-U*, 0.1 μM (dipalmitoleyl)-L-α-lecithin; *L-S*, 0.1 μM (dipalmitoyl)-L-α-lecithin; *LL-U*, 0.3 μM monopalmitoleyllecithin; *LL-S*, 0.3 μM monopalmitoyllecithin. Experimental details described in the text.

stimulation was approximately 0.3 μmole, compared to 0.1 μmole for lecithin. Above 1.0 μmole there was complete inhibition of the net oxygen uptake. The time-course for the oxidation of unsaturated and saturated lysolecithins is presented in Fig. 1.

The stimulation of net oxygen uptake by the lecithins followed a linear rate for the first 30 minutes, and the reactions appeared to be complete after 60 minutes. However, these results were somewhat variable, even when the same concentrations of lecithin were employed, and frequently the rate appeared linear for 120 minutes. The fact that lecithin is water-insoluble

[4] In a few experiments, the oxidation of lecithin and lysolecithin by the liver mitochondria of Sprague-Dawley rats was followed. In all these experiments, the oxygen uptake values ranged from 70 to 80 per cent of theory for complete oxidation to CO_2 and water.

made it difficult to ascertain the true molar concentration from experiment to experiment.

Lysolecithin, on the other hand, is water-soluble, and its effect on the time-course of the oxidation was reproducible within reason. An interesting observation on the lysolecithin oxidation curves was the inflection point occurring near 40 minutes (sharp increase in slope). At this point there was an apparently new effect which had a more pronounced slope than the initial rate and suggested that this effect might represent the stimulation of the oxidation of endogenous material by lysolecithin or a derivative. It must be said, however, that the net oxygen uptake observed with lecithin and lysolecithin cannot be interpreted as an oxidation of the fatty acids of these compounds, since only through the use of labeled lecithins or lysolecithins can their oxidation be established in an unequivocal manner.

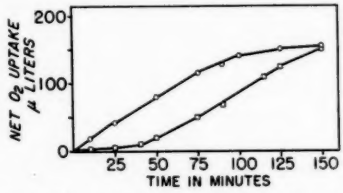

FIG. 2. Effect of lysolecithin on oxidation by mitochondria alone (○) and mitochondria plus supernatant fraction (□) of guinea pig liver. The experimental details are described in the text, and, in addition to the usual components, the reaction mixture contained 0.3 μM monopalmitoyllecithin.

Oxidation Studies with Guinea Pig Liver System—Since it has been found in the studies with rat liver mitochondria that the supernatant fraction of sucrose homogenates of the rat liver was necessary for the oxidation of lecithin and lysolecithin by the mitochondria, it was of interest to investigate the action of a similar system in the guinea pig liver.

Lecithin—It was found that lecithin did not stimulate oxidation with either the mitochondria or the reconstituted system of the guinea pig liver. This may be due to the failure of the intact lecithin to be "absorbed" by the mitochondrial system or to other unknown factors.

Lysolecithin—Several interesting observations were made in these initial experiments on guinea pig liver. As in the rat liver studies, lysolecithin was capable of stimulation of oxidation in the presence of the combined mitochondrial and supernatant fractions. Again there was an extended lag period prior to a large net oxygen uptake. However, in contrast to the rat liver response, in which both mitochondria and supernatant fraction were necessary, the guinea pig liver mitochondria alone were capable of demonstrating a large oxygen uptake with lysolecithin without any lag period. These results are presented in Fig. 2.

In an attempt to explain the rather ambiguous rôle of the supernatant fraction, the microsomal and soluble fractions were separated from the supernatant fraction and added separately to a mitochondrial system. It was noted that a definite inhibition of oxygen uptake occurred in the presence of lysolecithin with either the microsomal or soluble fraction. Upon recombining these fractions (microsomal and soluble), the stimulatory phenomenon occurred. The synergistic action of the microsomal and the soluble fractions is difficult to explain, particularly in view of the fact that Kornberg and Pricer (14) had found that the soluble fraction inhibited the phosphatidic synthesizing system in the microsomes.

Glycerylphosphorylcholine and Phosphorylcholine—Since the results thus far had shown that lysolecithin was capable of evoking a larger uptake of oxygen than that calculated from its own oxidation, it was thought that either this compound or some compound derived from lysolecithin might be "sparking" the oxidation of endogenous material and, in particular, fats.

An attempt was made, therefore, to determine whether the intermediate or intermediates might have structural features in common with lysolecithin. Among the possible compounds are glycerylphosphorylcholine, α-glycerophosphate, phosphorylcholine, and choline. It was expected that, if any of these compounds were to be an intermediate in lecithin metabolism, a stimulation of oxygen uptake would occur.

As shown in Fig. 3, GPC in the same molar concentration as lysolecithin stimulated oxygen uptake to an extent and at a rate comparable to that of lysolecithin. The net oxygen uptake of 200 μl. could not be accounted for by the oxidation of GPC, even if oxidized completely to CO_2 and H_2O, and must represent, for the most part, oxidation of endogenous material.

PC, in equimolar concentration, stimulated oxygen uptake to a larger extent than did GPC (Fig. 3). In subsequent experiments it was found that the PC effect was very consistent, whereas GPC at times failed in its ability to cause a net oxygen uptake.

Choline and Glycerophosphate—Choline alone had no effect on oxygen uptake. However, as shown in Fig. 4, in the presence of α-glycerophosphate, choline elicited the same response as did GPC. α-GP alone had a slight effect, which might have been due to some endogenous choline.

The compound which had the most consistent effect on the stimulation of oxygen uptake was phosphorylcholine, whereas the other compounds, GPC, lysolecithin, and GP plus choline, while definitely effective, did not consistently give large values of oxygen uptake. Thus, it seemed probable that the latter compounds were somewhat removed from the primary event responsible for the stimulation of oxygen uptake and that phosphorylcholine might be reacting more directly with some endogenous compound, possibly one containing fatty acids, which can be oxidized only in the presence of phosphorylcholine.

Effect of Concentration of PC on Stimulation of Oxygen Uptake—In the experimental results illustrated in Fig. 5, the PC concentration was varied to determine whether there was any relationship between substrate con-

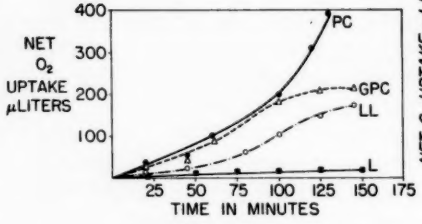

FIG. 3

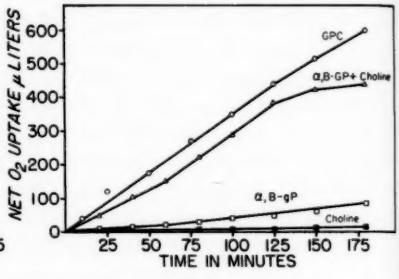

FIG. 4

FIG. 3. Influence of lecithin and derivatives on net oxygen uptake by guinea pig liver mitochondria. In addition to the usual components of the reaction medium (see the text), the following compounds were used: 0.5 μM phosphorylcholine (*PC*), 0.5 μM glycerylphosphorylcholine (*GPC*), 0.3 μM monopalmitoyllecithin (*LL*), 0.1 μM (dipalmitoleyl)-L-α-lecithin (*L*).

FIG. 4. Influence of glycerophosphate (□), choline (■), and glycerophosphate plus choline (△) on net O_2 uptake by guinea pig liver mitochondria. In addition to the usual components of the reaction medium (see the text), the following compounds were used: 0.4 μM glycerophosphate, 0.8 μM choline; glycerylphosphorylcholine (○), 0.6 μM, was used as a control.

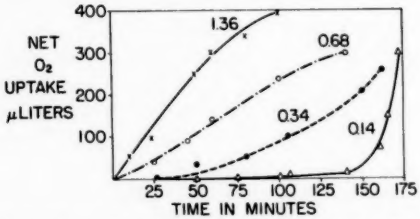

FIG. 5. Effect of varying concentrations of phosphorylcholine on net O_2 uptake in guinea pig liver mitochondria. In addition to the usual components of the reaction medium (see the text), phosphorylcholine was added in the concentrations indicated (in micromoles).

centration and the rate of oxidation. The results demonstrated that even in very small concentrations of PC (0.136 μmole) there was a large net oxygen uptake. The lag period noted with this quantity suggested that PC itself was not causing the stimulation of oxygen uptake, but rather that it was being converted to some intermediate compound which when present n sufficient quantity was being oxidized.

Relationship between Oxidation of Labeled Palmitic Acid and Stimulation Effect Caused by Phosphorylcholine—Implicit in the effects of lecithin, lysolecithin, GPC, GP plus choline, and PC on the oxygen uptake by mitochondria has been the assumption that the endogenous material being oxidized was possibly fatty acid which was derived from phospholipide, neutral fat, or cholesterol esters. In the initial experiments designed to test this hypothesis, palmitic acid-1-C^{14} was added to the guinea pig mitochondria along with some of the lecithin metabolites. Since other studies had demonstrated that the long chain fatty acids were primarily oxidized to CO_2 and not to acetoacetate (15), only the evolved CO_2 was assayed for radioactivity. The results of this study are described in Table I.

TABLE I

Effect of PC on Oxidation of Palmitic Acid

The experimental details are the same as those indicated in the experimental section, with the following additions: 0.68 μmole of PC, 0.17 μmole of palmitic acid-1-C^{14} (92,000 c.p.m.), and, where indicated, 6.0 μmoles of CoA. Reaction time, 3 hours at 37°. Each figure represents an average of a triplicate determination.

Substrate	Net O_2 uptake	$C^{14}O_2$	Per cent oxidation of palmitic acid to CO_2*
	μl.	*c.p.m.*	
PC + fatty acid + CoA	253	5600	6.1
" + " "	283	3900	4.3
Fatty acid	70	5240	5.7
" " + CoA	51	5000	5.4

* Calculated on the basis of the radioactivity in the added palmitate.

The data indicate that there was no apparent relationship between the stimulation phenomenon and the rate or amount of fatty acid oxidized. In fact, the amount of added fatty acid oxidized was depressed to the extent of approximately 30 per cent in the presence of PC. CoA reversed this inhibitory effect of PC and brought the oxidation values up to the level observed with either the fatty acid alone or the fatty acid plus CoA.

Influence of Phosphorylcholine on Oxidation of Tripalmitin—Inasmuch as PC had no apparent effect on the oxidation of palmitic acid (see Table I), it seemed possible that it might be affecting the oxidation of a fatty acid in an esterified form, *i.e.* a triglyceride. Consequently, in a mitochondrial system similar to that described for the palmitic acid studies, the effect of PC on the oxidation of tripalmitin-1-C^{14} was investigated.

The results in Table II indicate that PC was not affecting the oxidation of labeled tripalmitin to any significant extent. The fact that there was some oxidation to $C^{14}O_2$ (2 to 3 per cent) indicated that at least some of the

tripalmitin was oxidized. Furthermore, it cannot be ascertained whether the fatty acids of the tripalmitin were oxidized at random or whether only one of the fatty acids was oxidized to CO_2. In the latter case, the oxidation would be theoretically of the order of 9 to 19 per cent converted to labeled CO_2, which would be appreciable considering the fact that the true molar concentration of the tripalmitin is probably quite low in this system.

TABLE II

Effect of PC on Oxidation of Tripalmitin

The experimental details are the same as those indicated in Table I. Each cup contained 1.0 μmole of PC and 0.1 μmole of tripalmitin-1-C^{14} (85,000 c.p.m.). Each figure represents an average of a triplicate determination.

Substrate	Net O_2 uptake	$C^{14}O_2$
	μl.	*c.p.m.*
PC + tripalmitin	253	2470
Tripalmitin	67	2190

TABLE III

Effect of GPC on Oxidation of Palmitic Acid

The experimental details are the same as those indicated in Table I. Each cup contained 0.314 μmole of GPC, 0.34 μmole of palmitic acid-1-C^{14} (184,000 c.p.m.), and, where indicated, 6 μmoles of CoA. Each figure represents an average of a duplicate determination.

Substrate	Net O_2 uptake	$C^{14}O_2$	Per cent oxidation of fatty acid to CO_2*
	μl.	*c.p.m.*	
GPC + fatty acid + CoA	282	9880	5.2
Fatty acid + CoA	0	7200	3.8
" "	0	7500	4.0
GPC + fatty acid	0	7260	3.9

* Calculated on the basis of the radioactivity of the added palmitate.

Relationship between Oxidation of Palmitic Acid-1-C^{14} and GPC Stimulation Effect—The effect of glycerylphosphorylcholine on oxidation was not studied as extensively as that of PC, primarily because of the fact that consistent results of oxidation were not obtained. However, a limited number of experiments were performed with respect to the effect of GPC on the oxidation of palmitic acid, in a manner similar to the PC studies. These results are recorded in Table III.

As is evident from the above data, CoA appeared to influence both the stimulation phenomenon and the amount of labeled CO_2 produced. Previously CoA had not been implicated in the stimulation phenomenon with

GPC, but since the effects of GPC were quite variable it might be assumed that the effective CoA concentration in the liver varied from animal to animal. An interesting facet of this experiment was the finding that there was a significantly larger production of labeled CO_2 in the presence of GPC and CoA. This was quite different from the effects of PC, in which no influence of CoA on the oxidation of palmitic acid was noted. Furthermore, unlike PC, GPC did not depress the amount of oxidation of the fatty acid from the control value.

Relationship between Oxidation of Labeled Palmitic Acid and Stimulation Effect Caused by Lysolecithin—As in the case of GPC, only a limited study was made on the effects of lysolecithin on the oxidation of palmitic acid. These studies were performed in a manner similar to that described for

TABLE IV

Effect of Lysolecithin on Oxidation of Palmitic Acid-1-C^{14}

The experimental details are the same as those indicated in Table I. Each cup contained 0.322 μmole of (monopalmitoyl)-L-α-lecithin, 0.34 μmole of palmitic acid-1-C^{14}, and, where indicated, 6 μmoles of CoA.

Substrate	Net O_2 uptake	$C^{14}O_2$	Per cent oxidation of fatty acid to CO_2*
	μl.	*c.p.m.*	
Lysolecithin + fatty acid + CoA	156	8300	4.6
Fatty acid + CoA	0	7400	4.0
" "	0	7320	3.9
Lysolecithin + fatty acid	−101	4230	2.3

* Calculated on the basis of the radioactivity of the added palmitate.

PC, and the results are recorded in Table IV. CoA was necessary for the stimulation effect with lysolecithin. This was similar to the requirement of CoA for the GPC stimulation effect and suggests that there is relationship between the effects of lysolecithin and GPC on the stimulation of oxygen uptake.

SUMMARY

In the present studies conditions are described in which lecithin and certain of its derivatives caused an increased rate of oxygen uptake by liver mitochondria. In particular, lecithin and lysolecithin caused a stimulation of oxygen consumption in a rat liver mitochondrial system. When a guinea pig liver mitochondrial system was used, lysolecithin, glyceryl-phosphorylcholine, glycerophosphate plus choline, and phosphorylcholine, but not lecithin, caused a larger stimulation of oxygen uptake than could be expected for complete oxidation of these compounds.

The most effective and reproducible compound in this stimulation phenomenon was phosphorylcholine; however, it had no effect on the oxidation of added palmitic acid-1-C^{14} and tripalmitin-1-C^{14}. Lysolecithin and glycerylphosphorylcholine appeared to influence the oxidation of palmitic acid-1-C^{14}; coenzyme A was necessary, especially for the stimulatory effect of these two compounds on oxygen uptake by guinea pig liver mitochondria.

The significance of the increased oxygen uptake caused by these compounds has not been established, but it is suggestive of increased oxygen uptake by fatty acid derivatives.

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LECITHIN SYNTHESIS IN LIVER*

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Within the past few years considerable progress has been made in regard to the possible pathways of phospholipide synthesis in the liver. Kornberg and Pricer (1) have reported that in microsomal fractions of guinea pig liver α-glycerophosphate is a precursor of phosphatidic acid, which is formed by an acylation of glycerophosphate by coenzyme A derivatives of fatty acid. In a similar manner, Kennedy (2) has found that α-glycerophosphate in rat liver mitochondria fortified with the supernatant fraction is converted to a phosphatidic acid-like compound, which may then combine with choline to form a lecithin (3). In a preliminary report, Kornberg and Pricer (4) have indicated that phosphorylcholine can be incorporated as an intact unit into an unknown lipide fraction in guinea pig liver.

In the preceding paper (5), the stimulatory effect of certain lecithin derivatives, and in particular phosphorylcholine, on oxygen uptake in mitochondria was reported. The present work is a description of the results of an investigation on the incorporation of phosphorylcholine into the lipides of guinea pig liver.

EXPERIMENTAL[1]

Enzyme Preparation and Reaction Medium—The subcellular fractions were prepared as described in the preceding paper (5). The amount of enzyme used in all cases was the mitochondria or washed residue derived from 1 gm. (wet weight) of guinea pig liver. The reaction medium was that described previously (5). Wherever the term "sparkers" is indicated, it refers to the Krebs cycle intermediates.

Analytical Methods—Lipide phosphorus was determined by the method of King (6); choline, by the method of Glick (7).

Radioactivity measurements were made in the usual manner with either

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[1] The following abbreviations are used: P_i = inorganic phosphate; PC = phosphorylcholine; CoA = coenzyme A; ATP = adenosine triphosphate.

an end window counter for P^{32} determinations or a flow counter for C^{14} assays. When it was desired to distinguish between P^{32} and C^{14} in the same sample, a single layer of Parafilm (Marathon, Inc.) was placed over the sample, and it was counted in either a flow counter or an end window counter. This method reduced the emission C^{14} to negligible amounts, and there was no detectable absorption of the P^{32} β-emission. Specific activities are expressed in terms of counts per minute per mg. of lipide phosphorus.

Isolation of Lecithin—The isolation procedure was essentially as follows: To 3.0 ml. of reaction solution, 0.5 ml. of 7 per cent perchloric acid was added. The protein was removed by centrifugation and washed successively with 3 ml. of 1.0 per cent perchloric acid and 3 ml. of water and the washings discarded. All operations were carried out at room temperature. Further extractions of the residue with perchloric acid or water failed to remove any additional radioactivity. The residue was then extracted with 8 ml. of ethanol-ether (3:1) for 1 hour at room temperature. The insoluble material was removed by centrifugation and washed with 2 to 3 ml. of ethanol-ether (3:1), and the washings were added to the original extract. Since no further radioactivity was extracted with additional amounts of ethanol-ether or chloroform, the extraction of lipide material was assumed to be complete. The alcohol-ether extracts were concentrated to dryness at 37° under reduced pressure and in a stream of nitrogen. The residue was extracted three times with chloroform, and this extract was treated with acetone to precipitate the phospholipides. Duplicate determinations agreed within ±2 per cent, and the consistency of the results suggested that the method was valid for our purposes.

Lecithin was separated from the mixed phospholipides by a chromatographic procedure (8). Since only small quantities of material were available, proportionately smaller quantities of alumina were used. In a typical run, 50 mg. of mixed phospholipides in 1 ml. of ethanol were placed on a column containing 1.5 gm. of aluminum oxide. The column dimension was 8 × 190 mm., and the rate of flow of the eluate (95 per cent ethanol) was kept within the limits of 0.2 to 0.3 ml. per minute with a constant head of hydrostatic pressure.

Oxygen Uptake—Oxygen uptake measurements were performed in the usual manner. In the experiments in which oxygen uptake measurements were not necessary, a Dubnoff incubator was used with oxygen continuously flowing over the reaction medium. All experiments were performed at 37°.

Materials—Labeled inorganic phosphate was obtained from the Abbott Laboratories on an allocation from the Atomic Energy Commission, Oak Ridge, Tennessee.

Palmitic acid-1-C^{14} and tripalmitin-1-C^{14} were purchased from the Research Specialties Company[2] and Nuclear Instrument and Chemical Corporation, respectively.

Diglycerides were prepared by use of *Clostridium perfringens* as a source of enzyme (9) with yeast lecithin (10) and guinea pig liver lecithin as substrates. The characteristics of the diglycerides, D-1,2-dipalmitolein and D-1,2-dipalmitin, have been described previously (9). The diglyceride prepared from guinea pig lecithin had a hydrogen number of 225.9 and a saponification number of 301. The fatty acids of the guinea pig diglyceride were not identified, but it has been reported (11) that the α'-fatty acid is unsaturated, whereas the β acid is saturated.

L-α-(Dipalmitoyl)phosphatidic acid was kindly supplied by Dr. Erich Baer. DL-α-Glycerophosphate was prepared by the method of Bailly (12) and had a purity of 90 per cent by periodate titration. Calcium P^{32}-phosphorylcholine was prepared by the method of Riley (13) and was recrystallized from 50 per cent ethanol to constant specific activity. Eight batches were prepared and a typical analysis was as follows: calculated, P 9.46, N 4.25; found, P 9.52, N 4.25 per cent. Calcium ion was removed by use of an exchange resin IR-100 (potassium cycle). The calcium was completely removed without any loss of either radioactivity or phosphorylcholine.[3] C^{14}-labeled choline bromide (methylene-labeled) was obtained from Tracerlab, Inc., and converted to choline chloride. This was recrystallized to constant specific activity, diluted with non-labeled choline chloride, and used for the preparation of doubly labeled phosphorylcholine. The ratio of P^{32} to C^{14} is indicated in the results.

Results

Incorporation of Phosphorylcholine into Various Fractions of Guinea Pig Liver—Inasmuch as Kornberg and Pricer (4) had found that PC was incorporated into a lipide by the supernatant fraction of guinea pig liver, it was of interest to determine the relative amounts of PC incorporated into the three major subcellular fractions of the liver: the mitochondrial, microsomal, and soluble. The reaction medium employed for study of the microsomal and soluble fraction contained CoA and ATP, since it had been observed that these were necessary for the incorporation of PC (5). The results of these studies are reported in Table I.

As is evident from these data, the largest uptake of radioactivity into

[2] 1148 Walnut Street, Berkeley, California.

[3] As had been indicated previously (5), trace amounts of calcium had an inhibitory effect on the stimulatory effect of phosphorylcholine on mitochondrial respiration. In a similar manner, calcium, in a concentration of 7×10^{-5} M, inhibited the conversion of phosphorylcholine to lecithin.

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a lipide fraction occurred in the mitochondria. Since our previous studies with PC showed that it was capable of eliciting a large oxygen uptake in the mitochondria (5), it appeared that this uptake might be due to its incorporation into a lipide fraction. Although the other fractions contained activity, it was decided to use the mitochondrial fraction for further studies, primarily because of the stimulation effect.

Optimal Conditions for PC Incorporation—An attempt was made to ascertain the optimal conditions for PC utilization. In particular, the requirements for ATP and CoA were investigated.

On the basis of the results in Table II, it was evident that PC incorporation was enhanced 2-fold by the addition of the "sparkers." The

TABLE I

Incorporation of PC into Lipide Fraction of Cytological Elements in Guinea Pig Liver

Microsomal enzymes were derived from 20 gm. of liver and were taken up in 8 ml. of 8.5 per cent sucrose, and 0.5 ml. was added to each reaction flask. The soluble fraction had a total volume of 20 ml., and 2 ml. were added to each reaction flask. The medium used for the mitochondrial reaction was described previously (5). The medium for the microsomal and supernatant fractions was as follows: 0.2 ml. of 0.05 M ATP, 0.2 ml. of 0.005 M CoA, 0.1 ml. of 0.5 M $MgCl_2$, 0.2 ml. of 0.2 M cysteine, 0.5 ml. of 0.5 M phosphate buffer, pH 7.3, and 0.3 ml. of 0.5 M NaF. Total volume, 3 ml. Incubation time, 2 hours at 37°. 1.0 μmole of P^{32}-phosphorylcholine, specific activity 159,000 c.p.m. per μmole, was used in all the reactions.

Cytological fraction	Total P^{32} in lipide fraction
	c.p.m.
Mitochondrial	11,000
Microsomal	2,600
Supernatant	872

addition of ATP to the "sparkers" enhanced the incorporation over 3-fold, which suggested that oxidative phosphorylation was necessary for maximal incorporation. CoA did not appreciably affect the incorporation, although it is quite probable that the mitochondria contained sufficient CoA for maximal activity. This supports the previous finding (5) that added CoA also did not influence the stimulation phenomenon elicited by PC.

When varying concentrations of PC were plotted against specific activity of the lipide fraction (Fig. 1), there was a linear relationship between concentration and activity up to approximately 0.4 μmole and thereafter a diminution of activity. These results were obtained from reaction media incubated for 100 minutes. The specific activities were probably not indicative of the initial rate of incorporation over the entire range of concentration, and the decrease of specific activity at the higher concentrations might be due to a more rapid turnover of the lipide material.

Nature of Lipide from P^{32}-Phosphorylcholine Reaction—In order to determine the nature of the lipide which contained the labeled phosphorus, the

TABLE II

Conditions for Maximal PC Incorporation

All cups contained the standard reaction mixture and 1.5 ml. of washed mitochondria (45 to 65 mg. dry weight), as described previously (5), plus 1.0 μmole of PC. Other additions, where indicated, to this basic medium included 45.0 μmoles of citrate, 4.5 μmoles of α-ketoglutarate, 4.5 μmoles of malate ("sparkers"), 0.8 μmole of CoA, and 5.0 μmoles of ATP. Reaction time, 2 hours at 37°.

Additions	Amount of P^{32} incorporated	O_2 uptake
	per cent	*μl.*
None	2.7	1016
CoA	2.6	928
ATP	2.8	976
" + CoA	3.0	931
"Sparkers"	5.0	2263
CoA + "sparkers"	6.5	2444
ATP + "	9.1	2846
" + CoA + "sparkers"	9.1	2749

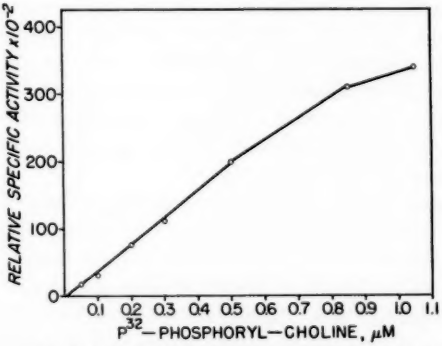

FIG. 1. Influence of phosphorylcholine concentration on the incorporation of P^{32} into lipide fraction of guinea pig liver mitochondria. Reaction conditions described in the text. Incubation time, 100 minutes at 37°. Relative specific activity is defined as counts per minute per mg. of lipide phosphorus.

phospholipides were isolated from reaction mixtures in the usual manner. In a typical experiment a crude phospholipide fraction, which had a specific activity of 3680 c.p.m. per mg. of lipide, was obtained from several reaction mixtures. This material, which weighed 50 mg., was chromatographed on an alumina column, and the purified lecithin, which represented 60 per cent of the phospholipide placed on the column, had a choline to phosphorus

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ratio of 1.03 (per cent P, 4.02; per cent choline, 16.2) and a specific activity of 6230 c.p.m. per mg. of lecithin. On the basis of previous evidence (8), it was assumed that this compound was a lecithin.

Effect of Tripalmitin-1-C^{14} on Incorporation of PC—In the studies carried out to this point, the PC was incorporated into endogenous material. It seemed of interest, therefore, to determine the nature of the endogenous material with which the PC was reacting.

Although PC had no apparent effect on the oxidation of labeled tripalmitin (5), it seemed possible that it might still be reacting with the tripalmitin, but the resultant phospholipide might not be oxidized because of an unnatural configurational position of the fatty acids (11). However, under exactly the same conditions of Table II for the maximal incorporation of phosphorylcholine into a lipide, no conversion of tripalmitin-1-C^{14} into lecithin was found.

Effect of Diglycerides on Incorporation of PC into Lipide Fraction—Although tripalmitin-1-C^{14} had no apparent effect on the incorporation of PC into a lipide fraction, it seemed possible that this was due either to the unavailability of this material as a substrate to the mitochondria or that the rate of reaction was very slow in comparison to the reaction of PC with the endogenous material.

It has been suggested that PC reacts with a diglyceride to form lecithin in the small intestine (14). Borgström (15) has found that the small intestine contains a lipase which specifically forms α,β-diglycerides. The suspected rôle of phospholipides in fat absorption from the small intestine and the similarity in the rates of turnover of lecithin in the liver and in the small intestine evoke the possibility that the mechanism of synthesis from glycerides is similar in both organs (14).

In the initial experiments, designed to test the influence of diglycerides, D-1,2-dipalmitolein was used as a substrate. When 0.3 μmole of this compound was added to the mitochondrial system in the presence of P^{32}-labeled PC, a large inhibition of oxygen uptake occurred. When the concentration was reduced to 0.1 μmole, however, there was no inhibition of oxygen uptake, and in some instances a small increase in oxidation was noted. However, of greater significance was the fact that, when two different concentrations of PC were used along with the diglyceride, there was an increase in the specific activities of the lipide material over that of the lipide isolated from reaction mixtures with PC alone. The results are presented in Table III.

There was approximately a 2-fold difference in the specific activities of the lipide fraction from the reactions with 1.0 and 0.5 μmole of PC. This is the ratio to be expected if the turnover of the diglyceride is dependent on the concentration of PC. However, the differences in specific activity

were not high relative to the total specific activity. Therefore, in order to emphasize the difference in specific activity and to show that it was due to the turnover of lecithin specifically, the lipide material from several reaction vessels was collected and the lecithin isolated and purified. In order to compare specific activities, the lecithin was isolated in exactly the same manner from mitochondrial reactions containing PC (1.0 μmole) and diglyceride (0.1 μmole) and those flasks containing PC alone.

The difference in the specific activity between reactions containing PC plus diglyceride and PC alone before and after column treatment was the same, indicating the difference was specifically related to the incorporation of the PC into lecithin. The ratio of choline to phosphorus in the fraction

TABLE III

Effect of Dipalmitolein on PC Incorporation

The usual reaction medium (5), with 1.5 ml. of washed mitochondria, contained, in addition, 0.1 μmole of dipalmitolein (DiP). Incubation time, 3 hours at 37°. Triplicate determinations.

Reactants	PC	Net O_2 uptake	Relative specific activity	Difference in specific activity	Difference incorporated
	μmole	*μl.*	*c.p.m.*	*c.p.m.*	*μmole P^{32}*
PC + DiP	1.0	330	27,300		
				4,165	0.012
"	1.0	273	23,135		
" + DiP	0.5	318	15,557		
				1,947	0.0057
"	0.5	274	13,610		

isolated from the column indicated that this material represented a lecithin type substance.

In subsequent experiments, it was found that D-1,2-dipalmitin was more effective than D-1,2-dipalmitolein in its influence on the incorporation of phosphorylcholine into a lecithin. Guinea pig diglyceride was as effective as dipalmitolein in its effect on this conversion.

Effect of Phosphatidic Acid on PC Incorporation into Lipide Fraction—Since phosphatidic acid formation has been indicated in the guinea pig liver by Kornberg and Pricer (1), it was considered that this may be an immediate precursor which reacted with PC to form lecithin. Although they had not found any evidence for the synthesis of phosphatidic acids in the mitochondria (1), the possibility remained that preformed phosphatidic acids were present. Accordingly, the influence of (dipalmitoyl)-L-α-phosphatidic acid on PC incorporation was studied. The results are indicated in Fig. 2.

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It is apparent that the rate of incorporation of PC was approximately doubled in the presence of the phosphatidic acid. Although this suggests that the phosphatidic acid is the immediate precursor of lecithin, no definitive evidence existed either that the PC was being incorporated into the lecithin fraction as a unit or that the fatty acids of the phosphatidic acid were likewise being incorporated.

In order to ascertain whether PC was incorporated as a unit into the lecithin fraction when phosphatidic acid was added to the reaction, the phospholipides were isolated from reaction mixtures which contained 5

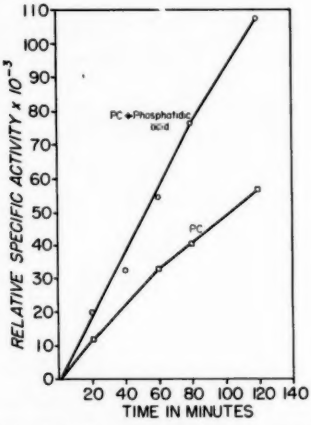

FIG. 2. Influence of phosphatidic acid on the incorporation of phosphorylcholine into the lipide fraction of guinea pig liver mitochondria. In addition to the usual reaction mixture described in the text, 1.5 ml. of washed mitochondria, 1.0 μmole of P^{32}-phosphorylcholine, and 0.2 μmole of phosphatidic acid were present. Incubation time, 2 hours at 37°.

times the usual amount of reactants, and the lecithin was isolated by chromatographic purification. These lipide fractions were analyzed for total fatty acids, choline, phosphorus, and radioactivity (see Table IV).

As indicated by these results, the phosphorylcholine was incorporated as a unit upon the addition of phosphatidic acid to the reaction. Since the previous data have indicated that phosphorylcholine incorporation into lipide was increased upon the addition of phosphatidic acid, it can be assumed that this was the result of lecithin synthesis.

Nature of Lecithin from Reaction between α-Glycerophosphate, P^{32},C^{14}-Labeled Phosphorylcholine, and Palmitic Acid-1-C^{14}—Since both Kornberg and Pricer (1) and Kennedy (2) have found that α-glycerophosphate was a precursor of phosphatidic acid, an attempt was made to determine

whether a reaction between α-glycerophosphate, P^{32},C^{14}-phosphorylcholine, and palmitic acid-1-C^{14} in a mitochondrial system would form lecithin labeled in the fatty acid, phosphorus, and choline. The lecithin was analyzed for total fatty acids, choline, phosphorus, and radioactivity (see Table V).

These results substantiate the fact that the material isolated by chromatography was lecithin, since the molar ratios of choline to phosphorus and fatty acid to phosphorus were approximately 1.0 and 2.0, respectively. As noted in the previous experiment (Table IV), the phosphorylcholine was incorporated as a unit. The labeled palmitic acid was ap-

TABLE IV

Lecithin Isolated from Reaction Mixture Containing Phosphorylcholine and Phosphatidic Acid

The lecithin was isolated from reaction mixtures which contained 5 times the usual amount indicated in Table II. In addition, the following components were present: P^{32},C^{14}-phosphorylcholine, specific activity of 300,000 c.p.m. per μmole and 0.75 μmole of phosphatidic acid. Incubation time, 3 hours at 37°. Triplicate determinations.

	Found	Theory
Choline, *μmole*	0.152	
Phosphorus, *μmole*	0.149	
Choline to P, *molar ratio*	1.02	1.00
$\frac{C^{14}\text{-Choline}}{\text{Total choline}}$, *μmole*	0.168	
$\frac{P^{32}\text{-P}}{\text{Total P}}$, *μmole*	0.167	
$\frac{C^{14}\text{-Choline}}{P^{32}\text{-Phosphate}}$, *molar ratio*	1.00	1.02

parently diluted by the endogenous fatty acid, since the labeled fatty acid was incorporated to about one-tenth the extent of the choline and phosphorus. These results showed again that phosphorylcholine was incorporated into a lecithin, but that very little palmitic acid-1-C^{14} was converted into a lecithin. Glycerophosphate appeared to have no influence on these reactions.

Incorporation of Inorganic Phosphate into Lipide Fraction and Influence of Choline on Its Incorporation—Inorganic phosphate has been shown by several investigators to be incorporated into phospholipides. It was of interest, therefore, to determine whether P^{32}-labeled inorganic phosphate was incorporated into the lipide fraction in the mitochondrial system. Choline was also added along with the phosphate in order to ascertain its effect on phosphate incorporation. Phosphorylcholine was used as a

standard of comparison for these reactions. The results are reported in Table VI.

Approximately 1.0 per cent of P_i was incorporated into a lipide fraction.

TABLE V

Nature of Lecithin Isolated from Reaction Mixture Containing P^{32},C^{14}-Phosphorylcholine, Palmitic Acid-1-C^{14}, and Glycerophosphate

The lecithin was isolated from reaction mixtures which contained 5 times the usual amount of medium indicated in Table II. In addition the following components were present: P^{32},C^{14}-phosphorylcholine, specific activity 300,000 c.p.m. per μmole, 4.3 μmoles; palmitic acid-1-C^{14}, specific activity 220,000 c.p.m. per μmole, 0.85 μmole; glycerophosphate, 15.6 μmoles. Incubation time, 2 hours at 37°. Triplicate determinations.

	Found	Theory
Total choline, *μmoles*	19.0	
" P, *μmoles*	18.5	
Choline to P, *molar ratio*	1.02	1.00
Fatty acids, *μmoles*	37.0	
" " to P, *molar ratio**	1.97	2.00
$\frac{C^{14}\text{-Choline}}{\text{Total choline}}$, *$\mu$mole*	0.229	
$\frac{P^{32}\text{-P}}{\text{Total P}}$, *$\mu$mole*	0.224	
$\frac{C^{14}\text{-Choline}}{P^{32}\text{-P}}$, *molar ratio*	1.02	1.02
$\frac{C^{14}\text{-Fatty acid}}{\text{Total fatty acid}}$, *$\mu$mole*	0.018	

* The fatty acids had a mean molecular weight of 285.

TABLE VI

Incorporation of P^{32}-Phosphate into Lipide Fraction and Influence of Choline on Its Incorporation

The reaction medium described previously, plus 1.5 ml. of washed mitochondria, contained in addition, where indicated, the following components: P^{32}-inorganic phosphate, specific activity of 5.6×10^6 c.p.m.; choline, 0.6 μmole; P^{32}-phosphorylcholine, 150,000 c.p.m. per μmole, 1.0 μmole. Incubation time, 2 hours at 37°. Triplicate determinations.

Substrate	Per cent of original P^{32} incorporated into lipide fraction
Inorganic phosphate	0.88
" " + choline	2.19
Phosphorylcholine	12.00

Of greater significance, however, is the finding that choline increased the incorporation of P_i more than 2-fold. Since choline did affect incorporation, it is quite probable that it is being phosphorylated, as suggested by Wittenberg and Kornberg (16). Phosphorylcholine was incorporated in the largest amount, which suggests that it is perhaps the immediate precursor to the phosphorylcholine moiety of lecithin.

DISCUSSION

The present findings that phosphorylcholine represents an important precursor and is converted as an intact unit into a lecithin by guinea pig liver mitochondria substantiate the earlier implications of the work of Kornberg and Pricer (4). These investigators reported that phosphorylcholine was incorporated into an unknown lipide fraction in extracts of guinea pig and rat liver. In the present investigation, optimal conditions for the incorporation of phosphorylcholine into lecithins required the presence of the Krebs cycle "sparkers," ATP and CoA.

It is of considerable interest that phosphatidic acid exerted a pronounced influence on the uptake of phosphorylcholine into lecithin. To a lesser extent diglycerides, such as dipalmitolein and dipalmitin, had a stimulatory effect on the uptake. On the basis of the observations of Kornberg and Pricer (1) that phosphatidic acid is formed in the microsomal fraction of guinea pig liver and the present indications that phosphatidic acid must play an important rôle in lecithin synthesis, it would appear that there is a reaction between this acid and phosphorylcholine. If such a reaction does occur, this would necessitate an exchange reaction between the entire phosphorylcholine unit and the phosphatidic acid, with the release of inorganic phosphate and the formation of lecithin. However, other routes to lecithin might be through a direct reaction between a diglyceride and phosphorylcholine or ATP plus diglyceride to form phosphatidic acid, which then reacts as above.

Recently, Kennedy (17) has reported that choline, by a pathway not involving phosphorylcholine as an intermediate, was incorporated into lecithin by rat liver mitochondria. A requirement for ATP indicated that this reaction was not the reverse of hydrolytic action. In a previous study, Kennedy (3) presented evidence which supported the possible conversion of glycerophosphate to a phosphatidic acid-like compound in rat liver mitochondria and a subsequent reaction of this compound with choline to form lecithin.

In attempting to explain the possible differences in the systems operative in rat liver and guinea pig liver, one must consider primarily that a species specificity does exist. Secondly, there remains the distinct possibility that more than one route for the synthesis of lecithin is available and there

is no necessity to invoke a single pathway. Although, in the systems described in this paper, phosphorylcholine was by far the best precursor for lecithin, it was observed that choline did influence greatly the incorporation of inorganic phosphate into lipide. Inasmuch as Wittenberg and Kornberg (16) have shown that the liver can form phosphorylcholine by the action of ATP on choline, this indicates a probable synthesis of PC in our systems. A significant point which emerged in these studies was that, unless the phosphorylcholine was freed completely of any metal ion by ion exchange chromatography, a definite inhibition of its uptake into lipide or its stimulatory effect on respiration (5) occurred.

SUMMARY

Phosphorylcholine has been shown to be incorporated as an intact unit and to a large extent into the lecithin of guinea pig liver mitochondria. ATP, CoA, and Krebs cycle intermediates are required for this conversion. Various diglycerides and phosphatidic acid, in particular, enhanced the incorporation of PC into lecithin. PC had little or no influence on the oxidation of palmitic acid-1-C^{14} or tripalmitin-1-C^{14}, nor was there any conversion of these labeled compounds into lecithins.

It was also demonstrated that P^{32}-inorganic phosphate was incorporated to a small extent into the lipide fraction, whereas choline stimulated the incorporation of labeled phosphate to an extent commensurate with the proposal that PC was synthesized by the mitochondria.

Some possible routes of synthesis of lecithin in this mitochondrial system are discussed.

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PURIFICATION AND CRYSTALLIZATION OF GLUCAGON

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Shortly after the discovery of insulin, Kimball and Murlin (1) postulated the existence of a hyperglycemic pancreatic factor, which they named glucagon. The factor was believed to be responsible for the initial transient hyperglycemia following parenteral administration of pancreatic extracts of insulin preparations. Several workers attempted to isolate and purify the active principle. A decade after Kimball and Murlin's original observations, Bürger and Brandt (2) were able to prepare a partially purified material having glucagon activity. They, however, experienced great difficulties in trying to obtain a substance free from insulin. The investigators hence concluded that insulin and glucagon must be closely related, especially since the chemical properties of the preparations they obtained were very similar to those of insulin itself. In more recent years other workers, Sutherland *et al.* (3) and de Duve and Vuylsteke (4), obtained small amounts of partially purified preparations having significant glucagon activity. Unfortunately, they were unable to obtain sufficient amounts of material to permit extensive characterization.

Our continuing interest in insulin and in its effects on carbohydrate metabolism led us to an interest in this substance, which had an opposite effect to insulin with respect to blood sugar levels. Characterization of the hyperglycemic factor with respect to both physical and biological properties and exploration of possible relationships to insulin necessitated isolation in an essentially pure form. The successful preparation of a crystalline material for which we accepted the name glucagon was announced previously (5) and is reported in detail in this paper.

Methods

The biological activity of glucagon was assayed in cats as described previously (6). The mouse convulsion assay was used for estimating the insulin activity of the various fractions.

The zone electrophoresis experiments were carried out essentially according to the procedure described recently by Fønss-Bech and Li (7). The starch[1] was washed thoroughly with the buffer employed for each particular experiment. The pH of the wet starch usually is somewhat different from

[1] Swan pure potato starch, distributed by Stein-Hall and Company, Inc., 1138 Merchandise Mart Plaza, Chicago, Illinois.

that of the buffer used to prepare the starch paste. It also drifts slightly during the course of the electrophoresis. The pH indicated for each experiment represents the average value of all the segment eluates.

Glucagon was hydrolyzed with glass-distilled 20 per cent HCl for 16 to 48 hours at 105° in sealed ampuls filled with nitrogen. Redfield's (8) method was used for the two-dimensional amino acid chromatography. Prior to analytical determinations, glucagon was dried for 48 hours at 40° *in vacuo* over phosphorus pentoxide.

Purification Procedure

An amorphous fraction obtained during the commercial purification of insulin was used as the starting material. It contained approximately 4 per cent glucagon and 7 per cent insulin. Denaturation of protein and hence inactivation of the biologically active components insulin and glucagon were prevented by using mild purification procedures. Thus, it was possible to follow the removal of insulin during purification by insulin assays. The hyperglycemic activity of the protein remaining dissolved in the supernatant fluids of Steps 1 to 3 was negligible.

Step 1. Acetone Fractionation—40 gm. of the starting material are dissolved in 1000 ml. of water by adjusting the pH to 3.5 with 10 per cent hydrochloric acid. The clear solution is cooled to 0–4° in an ice bath; 3.16 volumes of ice-cold acetone are added slowly from two or three separatory funnels with tips of small diameter. The suspension is equilibrated for 15 minutes, and the precipitate is collected by centrifugation; yield, approximately 20 gm. (dry weight).

Step 2. Fractional Precipitation at pH 4.3—Sodium acetate buffer is prepared by dissolving 90 gm. of anhydrous sodium acetate and 340 ml. of glacial acetic acid in 30 liters of water. The pH of this solution is adjusted to 4.3 with 10 per cent sodium hydroxide (approximately 40 ml.).

The material resulting from Step 1 is dissolved to prepare a 1 per cent protein solution. After adjusting the pH to 3.3 with 10 per cent hydrochloric acid, the solution is placed in a Visking cellophane membrane and dialyzed in the cold (4–7°) against the acetate buffer for 24 to 48 hours. The colloidal precipitate that formed slowly during dialysis is collected by centrifugation at high speed (30 to 50 minutes at 20,000 × *g*). It is resuspended in approximately 250 ml. of water and is dialyzed for 24 hours against 0.025 N hydrochloric acid, and thereafter for 24 to 48 hours against water at 4–7°. The final solution contained 6 to 6.5 gm. of protein.

Step 3. Fractional Precipitations at pH 2.5—All dialysis operations described in this step are carried out in the chill-room at 4–7°. The dilute acid used is 0.025 N hydrochloric acid. The phosphate solution is prepared by dissolving 15 gm. of KH_2PO_4 per liter of water and adjusting the pH to 2.5 with hydrochloric acid.

The solution resulting from Step 2 is diluted with water (approximately 350 ml.) to obtain a protein concentration of 1 per cent. After adjusting the pH to 2.5 with 0.2 per cent hydrochloric acid, the solution is placed in a Visking cellophane membrane and is dialyzed for 24 hours against 30 liters of the phosphate solution. The colloidal precipitate is, as in Step 2, collected by centrifugation at high speed and is redissolved in 500 ml. of dilute acid. The solution is dialyzed, first for 3 hours against 0.025 N hydrochloric acid and thereafter for 24 hours against the phosphate solution. The collected precipitate is redissolved and dialyzed once more in the same manner. The salt is removed from the final precipitate by dissolving it in 250 ml. of dilute acid and by extensive dialysis (40 to 48 hours) of the solution against 0.025 N acid followed by water. The solution, virtually free of phosphate, is lyophilized. Yield, 1.3 to 1.8 gm. of a slightly yellow powder.

Step 4. Crystallization of Highly Purified Glucagon—1 gm. of the lyophilized material from Step 3 is dissolved in 75 ml. of water, 25 ml. of 4 M urea are added, and the pH of the mixture is adjusted to 8.9 with 0.2 per cent sodium hydroxide. The volume is brought to 150 ml. with 0.1 M glycine buffer of pH 8.5 and the solution (pH 8.6) is allowed to stand in the refrigerator. Crystallization starts after a few hours and is completed within 24 to 48 hours. The crystals are collected by gentle centrifugation in a clinical centrifuge; they are washed three times with a cold 0.01 per cent sodium chloride solution and dried subsequently *in vacuo* over phosphorus pentoxide. The colloidal precipitate which has formed during the crystallization process is removed by centrifugation at high speed. It contains 40 to 50 per cent glucagon, part of which can be recovered in crystalline form by repeating the crystallization step.

Recrystallization of glucagon is performed by using essentially the same procedure as described above. For example, 100 mg. of crystalline glucagon are dissolved in 14.5 ml. of water by adjusting the pH to approximately 11. 870 mg. of urea and 29 mg. of glycine are added. The solution is centrifuged and the clear supernatant fluid is readjusted to pH 8.5 with a small amount of 10 per cent hydrochloric acid. This operation precipitates some of the protein in amorphous form; its transformation into crystals, however, takes place upon standing in the refrigerator for 24 to 48 hours. The crystals are collected, washed, and dried in the described manner. Washing of once, or especially twice, recrystallized glucagon with a cold 0.01 per cent sodium chloride solution is essential, since the fragile crystals are often transformed into a gel if washed with electrolyte-free water. The specific biological activity of the residual protein, isolated from the mother liquor after recrystallization of glucagon, is identical with that of the crystals. Thus, within the limits of the biological test, no additional purification is accomplished by repeated recrystallization. The

protein recovery and distribution of biological activities for each purification step are summarized in Table I.

Characterization and Properties of Glucagon

The glucagon crystals belong to the isometric system and appear as the sharply defined rhombic dodecahedra shown in Fig. 1.

TABLE I

Distribution of Protein and Biological Activity in Inactive and Active Fractions of Each Purification Step

Purification step	Inactive protein fraction			Active protein fraction				Over-all recovery of activity
	Recovered protein	Glucagon content*	Insulin	Recovered protein	Glucagon content*	Insulin	Stepwise recovery of activity	
	per cent	*per cent*	*units per mg.*	*per cent*	*per cent*	*units per mg.*	*per cent*	*per cent*
Acetone fractionation	50	0.6–1	2.5	50	7–8	1.0	87	87
Fractional precipitation in acetate buffer at pH 4.3	65–70	1 –1.3	1 –1.3	30–35	20	0.5	86	75
Fractional precipitation in phosphate solution at pH 2.5								
1st dialysis	70–75	1.5–2	0.7 –0.8	25–30				
2nd "	10	2	0.7	90				
3rd "	3	2	0.5	97	50–60	~0.05	55	41
Crystallization	29†	1	0.2 –0.3					
"	48‡	40 –50	~0.05	23	100		46	19

* Based on the assumption that crystalline glucagon is 100 per cent pure.

† Recovered from supernatant fluid.

‡ Colloidal precipitate formed during crystallization.

The crystalline powder is generally white, but occasionally has a bluish tint owing to trace contaminations of copper and cobalt ions. Emission-spectroscopic analysis revealed that the glucagon crystals obtained by the crystallization described contain less than 0.01 per cent of zinc. The moisture content is on the average 12.8 per cent; it can be removed by drying the crystals for 20 to 24 hours *in vacuo* at 100° over phosphorus pentoxide. The crystalline protein is relatively insoluble in water, especially in the presence of electrolytes. It, however, is soluble at alkaline and acid pH values, *e.g.* between pH 9.5 and 10.5 and below pH 4. The solubility is partly dependent on the amount and type of electrolyte present. Highly purified glucagon tends to form fibrils rather easily in acid aqueous solution. Fig. 2 shows an electron micrograph of glucagon fibrils.

The fact that no additional purification was accomplished by repeated recrystallization suggested that the crystalline glucagon preparations were of respectable purity. In addition, end-group analysis provided evidence that the crystalline product contained no detectable amounts of contaminating proteins. Sanger's dinitrophenyl procedure (9) yielded only 1 *N*-terminal residue, namely didinitrophenyl-histidine. The purity of glucagon has also been studied by zone electrophoresis with starch as a supporting medium. Crystalline and recrystallized preparations were

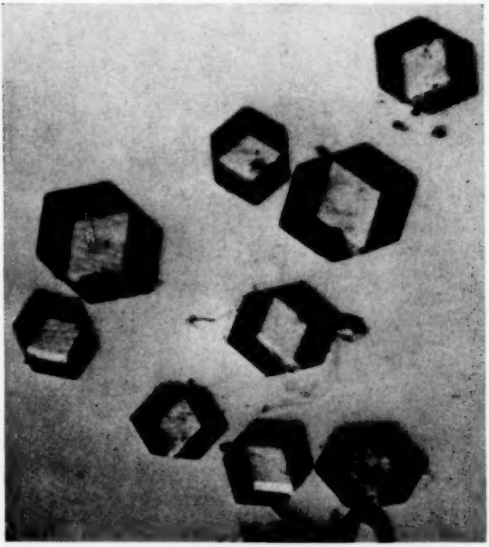

FIG. 1. Photomicrograph of glucagon crystals (100 ×)

tested under varying experimental conditions. Curve A of Fig. 3 represents the distribution of 6 mg. of once recrystallized glucagon after electrophoresis for 40 hours (160 to 170 volts, 13 to 15 ma.) at pH 8.8 in starch buffered with 0.075 M glycine. It is evident that the protein separated into two distinct peaks. The faster moving component comprises approximately 11 per cent of the total glucagon applied. The slight adsorption of protein at the point of application is probably due to the relatively low pH of this particular experiment. Adsorption did not occur in a similar experiment at pH 9.86 (Curve B). The more alkaline pH caused the protein to migrate somewhat faster, but had no effect on the proportion or separation of the two components. Repeated recrystallization of glucagon failed to remove the small constituent. Curve C illustrates the distribu-

tion of a glucagon sample recrystallized three times subjected to zone electrophoresis at pH 10.3 for 40 hours. The curve demonstrates that the two components are still present in approximately the same proportion.

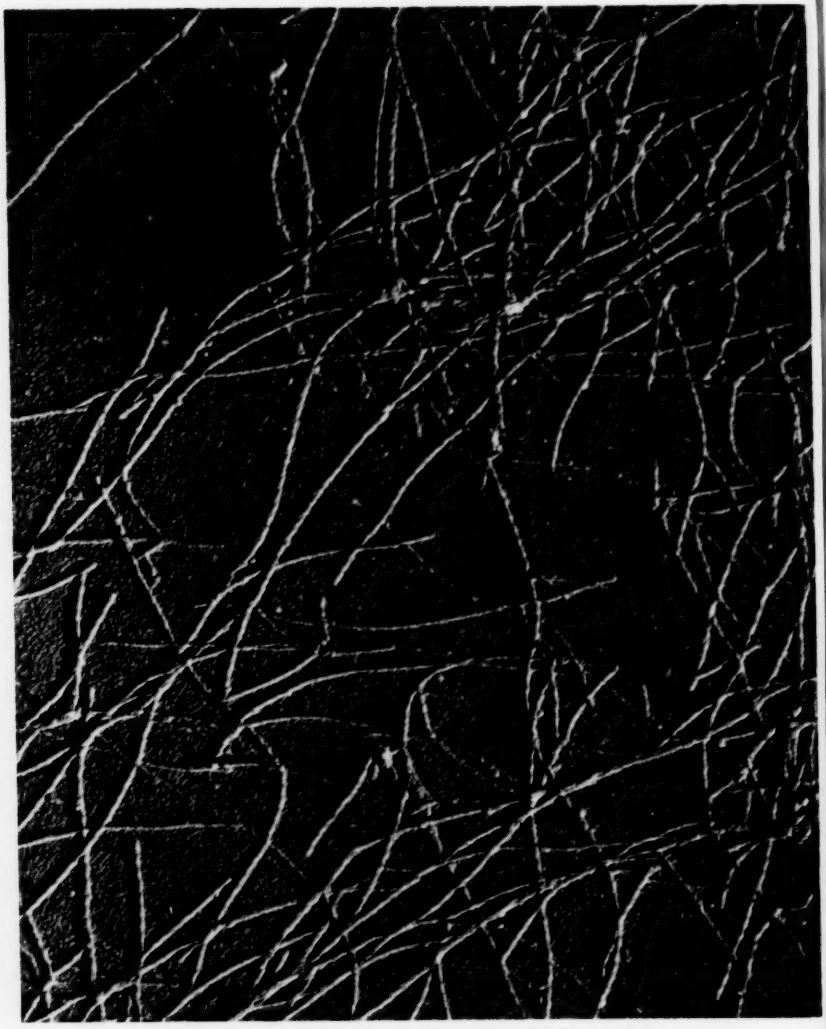

FIG. 2. Electron micrograph of glucagon fibrils (20,000 ×)

Analysis of the minor component reveals that its qualitative amino acid composition is identical with that of the main peak and the crystalline protein itself. It is biologically about half as active as crystalline glucagon. These data suggested that the faster moving protein might be derived from glucagon itself, rather than being an extraneous impurity. This hypothesis was tested by reelectrophoresis of the protein isolated from the main

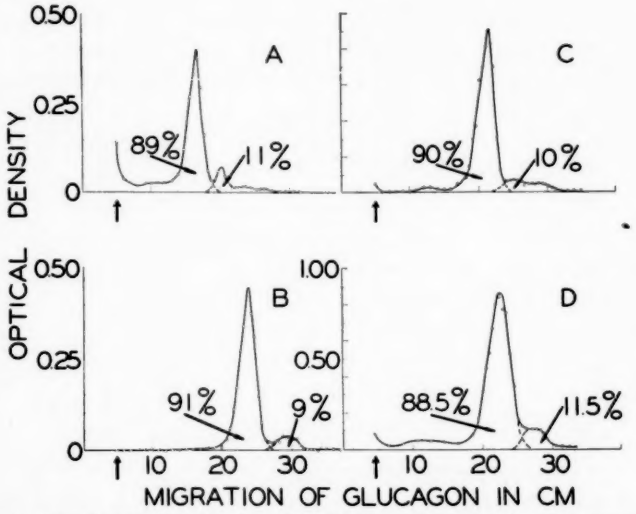

FIG. 3. Zone electrophoresis of crystalline glucagon on starch. The arrows indicate the point of application of the protein. Curve A, glucagon recrystallized once, 0.075 M glycine buffer, pH 8.80; Curve B, glucagon recrystallized once, 0.075 M glycine buffer, pH 9.86; Curve C, glucagon recrystallized three times, 0.05 M Na_2CO_3 solution, pH 10.3; Curve D, rerun of crystalline glucagon isolated from main peak (see Fig. 4), 0.075 M glycine buffer, pH 9.68.

peak of two large scale experiments, 20 mg. of crystalline glucagon being employed for each run. The electrophoresis (150 volts, 13 to 15 ma.) was carried out in a 60 cm. long trough at pH 10.2. The starch was washed with a glycine buffer (0.075 M) of pH 11.5. The time of electrophoresis was extended to 65 hours to insure separation of the two constituents. The shaded area in Fig. 4 indicates the portion which was used for the isolation of the main component. The recovered protein was crystallized and its electrophoretic behavior studied under the same conditions as employed for Curve B (Fig. 3). The result recorded in Curve D of Fig. 3 shows that reformation of the faster moving constituent has taken place in

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about the same proportion. This result is consistent with the interpretation that the small component is derived from glucagon itself. The evidence obtained from these experiments suggests strongly that crystalline glucagon is a protein of high purity, although further studies are necessary to prove its purity conclusively.

The elementary composition of twice recrystallized glucagon is summarized as follows: C 50.11, H 6.42, S 0.77, N 17.45 per cent. Glucagon may be distinguished from insulin by its relatively high nitrogen and low sulfur content. It should be noted, however, that all the analytical values presented are comparable to those found for most typical proteins. Two-

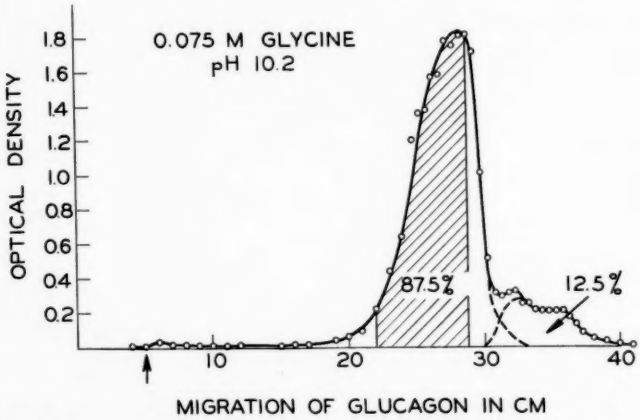

FIG. 4. Large scale zone electrophoresis of crystalline glucagon on starch. The arrows indicate the point of application of the protein.

dimensional paper chromatography of glucagon hydrolysates revealed the following components: methionine, tryptophan, arginine, histidine, lysine, phenylalanine, tyrosine, leucine, valine, alanine, threonine, serine, glycine, aspartic acid, glutamic acid, and ammonia. The amino acid composition of glucagon differs significantly from that of insulin with respect to several amino acids. Cystine, proline, and isoleucine could not be detected by chromatographic procedures even when relatively high concentrations of glucagon hydrolysates were used. The protein, however, contained considerable amounts of methionine and tryptophan; both of these amino acids are absent in insulin. Quantitative determinations of methionine showed that it accounts for all the sulfur found in crystalline glucagon; this confirms the finding that no other sulfur-containing amino acids are present.

Parenteral administration of crystalline glucagon produces a rapid increase in blood sugar, which is presumably due to the liberation of glucose from glycogen stores in the liver (10). The minimal effective dose is dependent on factors such as animal species, route of administration, and nutritional status. The hyperglycemic response of near minimal doses of crystalline glucagon in cats, rabbits, and mice is illustrated in Figs. 5 and

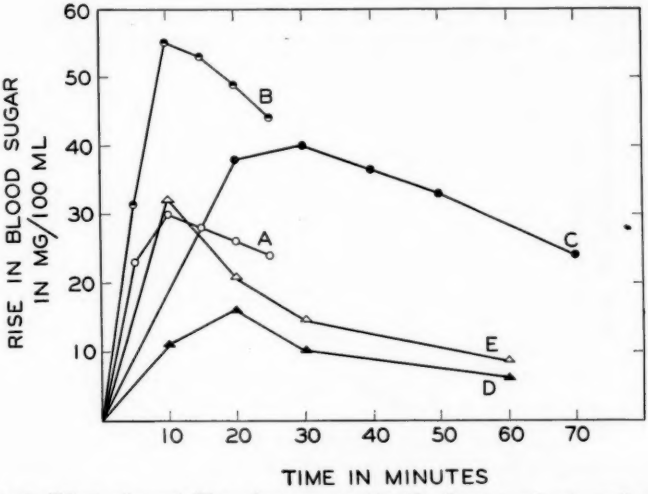

FIG. 5. Effect of crystalline glucagon on the blood sugar of cats and rabbits. Curve A, average response of seven cats (fasted for 16 hours) to intravenous injection of 0.05 γ of glucagon per kilo of body weight. Curve B, average response of eight cats (fasted for 16 hours) to intravenous injection of 0.3 γ of glucagon per kilo of body weight. Curve C, average response of seven cats (fasted for 16 hours) to subcutaneous injection of 10 γ of glucagon per kilo of body weight. Curve E, average response of thirteen non-fasted rabbits to intravenous injection of 1 γ of glucagon per kilo of body weight. Curve D, average response of thirteen non-fasted rabbits to subcutaneous injection of 1 γ of glucagon per kilo of body weight.

6. The blood sugar Curves A, B, and C of Fig. 5 illustrate the hyperglycemic responses in cats obtained with varying amounts of glucagon injected intravenously and subcutaneously. The curves clearly show that glucagon is considerably less effective when administered by the subcutaneous route than when given intravenously. Subcutaneous injection of glucagon up to 3 γ per kilo of cat body weight produced no significant hyperglycemia, whereas by intravenous administration as little as 0.05 γ per kilo caused a blood sugar rise of approximately 30 mg. per cent. This difference in activity is not observed in the rabbit. The hyperglycemia achieved

by 1 γ of crystalline glucagon per kilo, given by either route, is of the same order of magnitude. Fig. 5 reveals, furthermore, that the rabbit is less sensitive to glucagon than the cat. The former requires about 10 to 15 times more glucagon to achieve a comparable rise in blood sugar. The sensitivity appears to differ from species to species. White mice of a standard strain are relatively insensitive to glucagon. The blood sugar rise produced by a dose of 0.1 γ per mouse (25 to 30 gm.) is not significant

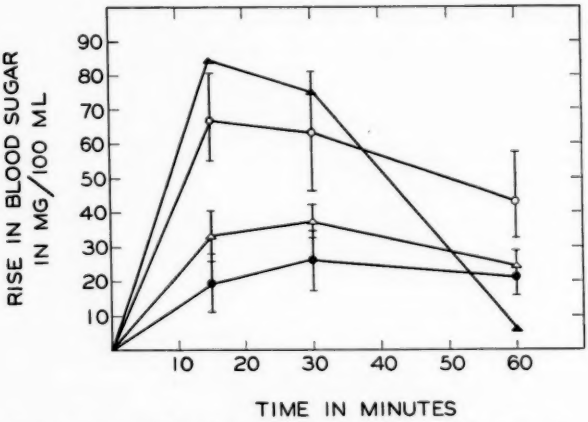

FIG. 6. Effect of crystalline glucagon on the blood sugar of mice. ○, average response of ten mice (fasted for 16 hours) to subcutaneous injection of 2 γ of glucagon per mouse (20 to 30 gm.). ▲, average response of eight non-fasted mice to subcutaneous injection of 2 γ of glucagon per mouse. △, average response of twelve mice (fasted for 16 hours) to subcutaneous injection of 0.1 γ of glucagon per mouse. ●, average response to injection of insulin-diluting fluid as a control. The hyperglycemia probably is due to the increased temperature (37°) to which all mice were subjected immediately after injection.

even when injected intravenously. The same dose on a kilo basis (3 to 4 γ per kilo) would cause a pronounced hyperglycemia in cats or rabbits. As illustrated in Fig. 6, a significant hyperglycemia can be obtained in mice by administering larger amounts of glucagon, *e.g.* 1 to 2 γ per mouse. Anesthetized cats not only are more sensitive to glucagon than either rabbits or mice, but they also show less variation in sensitivity. It was found that the standard error of the mean in mice for groups of fifteen to seventeen animals varied between 20 and 30 per cent, whereas, for the same number of cats, it never exceeded 10 per cent.

In regard to stability it is evident from earlier work (2, 10) that glucagon is more resistant to alkali than insulin. This difference can be explained

on the basis of the amino acid composition of the two proteins. The insulin monomer (mol. wt. 6000) contains 3 molecules of cystine, an amino acid that is very susceptible to alkali. Even partial rupture of the disulfide linkages results in a total loss of the hypoglycemic activity (11, 12). The absence of cystine in glucagon is probably responsible for its relatively high stability in the presence of alkali. Although alkaline glucagon solutions are slowly inactivated as shown in Fig. 7, they can be kept under refrigeration for at least a few weeks without noticeable loss in biological activity. Glucagon dissolves readily by adding small amounts of alkali to

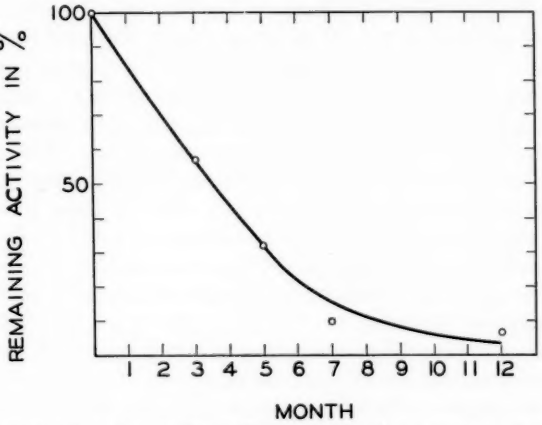

FIG. 7. Inactivation of crystalline glucagon in alkaline solution. Loss in biological activity of a 0.02 per cent glucagon solution in 0.1 M glycine buffer at pH 9.9 kept at 4° for 12 months.

crystal suspensions. These solutions can be kept at alkaline pH values of 10.5 to 11.5 for several hours without endangering the biological activity. Like insulin, glucagon appears to be more stable at acid pH values. A 0.1 per cent glucagon solution in insulin-diluting fluid at pH 3 was kept in the chill-room (3–4°) for 7 months without ensuing loss of hyperglycemic activity. Dry crystalline glucagon is relatively resistant to heating. Its biological activity remained unchanged after a heating period of 48 hours *in vacuo* at 105°.

DISCUSSION

Many of the earlier reports dealing with the action of crude insulin preparations suggested that the hyperglycemic effect might be an inherent property of insulin (13, 14) or be due to an artifact created during its

manufacture (15). Bürger and Brandt (2) were first to study the properties of purified glucagon preparations. Their data suggested, in agreement with Kimball and Murlin's hypothesis, that the hypo- and hyperglycemic activities of pancreatic extracts are due to two different substances. Bürger and Brandt's preparations were active in rabbits at a dose level of 20 γ per kilo of body weight (given intravenously). It seems unlikely, however, that the purity of these samples exceeded 10 per cent, since 1 to 2 γ of crystalline glucagon per kilo of rabbit produced a comparable response. On this basis it is not surprising that the properties of crystalline glucagon described in this paper differ significantly in many respects from those described by the German investigators. Their preparations, for example, contained 2.7 per cent sulfur, whereas crystalline glucagon contains only 0.77 per cent. The low sulfur content of glucagon is explained by the absence of cystine which is present in considerable amounts in impure glucagon preparations or in insulin. Furthermore, the pK of glucagon is, contrary to Bürger and Brandt's observation, higher than that of insulin. Preliminary experiments indicated that the isoelectric region of glucagon is approximately between pH 7.5 and 8.5. The chemical and physicochemical properties of the crystalline protein prove conclusively that glucagon is indeed an entity distinctly different from insulin. The possibility of its being a degradation product of the latter is eliminated by the amino acid composition of glucagon.

Mohnike and Boser (16) recently reported the preparation of a crystalline hyperglycemic, glycogenolytic substance. It appears to be a nucleoprotein and differs markedly from the crystalline glucagon described in this paper. Glucagon is free of carbohydrate and has all the properties of a simple protein. It contains methionine and threonine, two amino acids which are absent in the nucleoprotein. On the other hand, isoleucine and proline, two constituents of Mohnike and Boser's preparation, are missing in glucagon. The difference in biological activity between the two proteins is remarkable. 500 γ of Mohnike and Boser's preparation are necessary to achieve a minimal blood sugar rise in a rabbit weighing 3.5 kilos; a comparable response is obtained with as little as 2 to 3 γ of crystalline glucagon. Moreover, the biological activity of the nucleoprotein disappears rapidly, even when stored as a dry powder, whereas glucagon under these conditions is stable. In view of these facts, it is concluded that the two substances are entirely different.

Weitzel *et al.* (17) in a recent publication reported on the relationship between zinc content and hyperglycemic effect of several crystalline insulin preparations. The hyperglycemic activity of insulin, within certain limits, appeared to be a function of the zinc content. Insulin samples of low zinc content showed little or no hyperglycemic activity; however, considerable

biological activity was noted for preparations containing 0.9 to 1.4 per cent of zinc. Removal of part of the zinc from such samples produced insulin crystals essentially free of hyperglycemic activity. Based on these data, the investigators speculated that the hyperglycemic factor might possibly be inactive by itself, gaining its full potency only through formation of a complex with zinc and insulin. This hypothesis, however, is unlikely, since the biological activity of crystalline glucagon, containing at the most traces of zinc and insulin, is higher than that of any preparation described in the literature. Addition of either one or both of these agents has no potentiating effect on the biological activity. The protein, as far as it is known, contains no non-protein prosthetic groups. The hyperglycemic effect of glucagon, therefore, must be an inherent property of the polypeptide. Although this work clearly demonstrates that the formation of a complex with either insulin or zinc is not a prerequisite for biological activity, the possibility that these substances may form association products or complexes as described by Tanford and Epstein (18) is not eliminated.

Based on quantitative determinations carried out for some of the amino acids, it is possible to make an estimation with respect to the molecular weight of glucagon. Two-dimensional paper chromatography of dinitrophenyl-glucagon hydrolysates revealed no free histidine. Furthermore, no separate ninhydrin-positive spot, possibly due to colorless imidazole-dinitrophenyl-histidine was detected. The *N*-terminal histidine residue is therefore the only one occurring in the polypeptide chain and accounts for all of the 3.7 per cent histidine found in crystalline glucagon. This conclusion is in agreement with molecular ratio determinations and sequence studies presently in progress. The molecular weight of glucagon calculated from these data is 4200; the same value was obtained on the basis of the methionine content. This suggests that the polypeptide is composed of approximately 28 to 32 amino acid residues.

SUMMARY

The isolation and crystallization of glucagon, the hyperglycemic-glycogenolytic factor of the pancreas, are described. Physical and chemical studies indicate that the crystalline protein is of high purity. The amino acid composition revealed that glucagon is a distinct entity with no apparent relationship to insulin. Histidine is the *N*-terminal amino acid. Glucagon is entirely different from the hyperglycemic substance recently described by Mohnike and Boser. Crystalline glucagon virtually free of zinc is biologically highly active; its activity is not potentiated by addition of either zinc or insulin.

We are pleased to acknowledge the assistance of Dr. E. O. Davisson and

Mr. R. Gamewell in providing the picture of glucagon fibrils. We are indebted to Dr. H. A. Rose for photographing and characterizing the crystals.

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PREPARATION OF PHOSPHATIDYL ETHANOLAMINE FROM SOY BEAN PHOSPHATIDES*

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In a previous paper (1), it has been shown that phosphatidyl ethanolamine can be isolated from the alcohol-insoluble fraction of soy bean phosphatides. Fractions obtained by counter-current distribution of this material were treated with lead acetate to precipitate an inositol-containing phosphatidic acid and other substances, leaving phosphatidyl ethanolamine in solution. The utility of this procedure as a source of phosphatidyl ethanolamine is lessened by requirements of special apparatus and the relatively small capacity of the counter-current distribution step. For large scale work a long and complicated operation in large separatory funnels must be substituted. Since soy bean phosphatides are readily available, simplified methods for the preparation of phosphatidyl ethanolamine from this source were investigated. The present paper describes a study of two such methods by which phosphatidyl ethanolamine of 85 per cent or higher purity has been prepared. Preparation A makes use of an extraction procedure based on the counter-current distribution previously used. In Preparation B, treatment of a chloroform solution with methanol is substituted as the initial step.

EXPERIMENTAL

Analytical Methods—Phosphatides were analyzed by methods similar to those used in earlier work (1). Phosphorus was determined according to Truog and Meyer (2) on samples ashed with magnesium nitrate. Total nitrogen was determined by a micro-Kjeldahl procedure. Choline was measured by the reineckate method of Glick (3). Samples for nitrogen determination by the periodate method of Burmaster (4) were hydrolyzed by refluxing for 24 hours with 10 ml. of 2 N H_2SO_4. For use in the inositol assay (5) samples were hydrolyzed with 20 per cent HCl at 120° for 16 hours. In sugar determinations, samples were refluxed for 20 hours with 2 N H_2SO_4, filtered, and neutralized. This hydrolysis is satisfactory for samples from which free sugars have been removed. With the acetone-insoluble phosphatides which still contained free sugars, a milder hydroly-

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sis, as described in a previous paper, was used (6). Sugar was determined by Somogyi's procedure (7).

Preparation of Alcohol-Insoluble Fraction—The alcohol-insoluble fraction of soy bean phosphatides used in this work was prepared as previously described (1). Commercial soy bean lecithin was first stirred with acetone, cooled to 10°, and filtered on a Büchner funnel. The insoluble material was dissolved in hexane, precipitated by pouring into acetone, cooled to 10°, and filtered. The precipitation of this insoluble material from hexane solution was repeated three times. In the first two precipitations, the mixture was cooled to 10°; the last two precipitations were carried out at room temperature. Acetone was removed from the insoluble material under vacuum. From 768 gm. of commercial lecithin, 502 gm. of acetone-insoluble material were obtained. This material (493 gm.) was divided into three portions and each portion was extracted in a Waring blendor[1] with 400 ml. of absolute alcohol. After filtration on a Büchner funnel, the insoluble material was divided into two portions, and the extraction was repeated five times at room temperature and five times with absolute alcohol warmed to 55–60°. The wet filter cake after the last extraction weighed 334 gm. Drying of a small sample indicated that it contained 36 per cent alcohol. Hexane, water, and additional alcohol were added to the remainder of the wet cake to give a solution containing 3 ml. of 50 per cent alcohol and 3 ml. of hexane for each gm. of material. This mixture was shaken gently in a separatory funnel, centrifuged to break the emulsion, and separated into two layers. The hexane layer was extracted twice more with 50 per cent alcohol, since previous work (6) had shown that the free sugars were removed from phosphatides by this procedure. The hexane solution was then evaporated to about three-fourths of its original volume and added to 5 liters of acetone. After filtration on a Büchner funnel, the insoluble material was dried under vacuum. Alcohol-insoluble phosphatides low in sugar thus were obtained in a yield of 166 gm. Analytical data on this material are shown in Table I.

Preparation A—This preparation is a simplified variation of the countercurrent distribution procedure previously used (1). 50 gm. of the alcohol-insoluble fraction were dissolved in 175 ml. of hexane previously equilibrated with 95 per cent methanol. The hexane solution was extracted ten times with 400 ml. portions of the 95 per cent methanol equilibrated with hexane. Methanol Extracts 1 through 5 and 6 through 10 were combined to give two solutions which were then processed separately. Each solution was extracted with 75 ml. of the hexane layer, filtered to remove a slight

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the United States Department of Agriculture over other firms or similar products not mentioned.

cloudiness, and partially evaporated under vacuum to remove hexane. The solutions became cloudy on evaporation but became clear again upon dilution to their original volumes with methanol. An excess of lead acetate solution (30 gm. of lead acetate made up to 100 ml. with water) was added. For the combined Extracts 1 through 5, 25 ml. of lead acetate solution were required; for the combined Extracts 6 through 10, 15 ml. Precipitated lead salts of phospholipides were removed by filtration.

Excess lead ion was removed from the filtrates by passing carbon dioxide gas from a cylinder into the solutions. The solutions were then filtered to remove lead carbonate. This procedure has been found to be much more satisfactory than the 10 per cent acetic acid wash previously described (1).

TABLE I

Analysis of Phosphatide Fractions

Fraction	Yield*	P	N	Choline N	Bur-master N	Inositol	Sugar
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Acetone-insoluble soy bean phosphatides		3.06	1.12	0.44			10.5†
Alcohol-insoluble low sugar		3.50	0.73	<0.001		7.45	2.74
Preparation A, Extracts 1–5	5.1	3.75	1.56			0.06	3.21
" " " 6–10	3.3	3.72	1.72			0.08	1.74
" B	19.8	3.61	1.62		1.37	0.02	2.17

* Per cent of product based on weight of alcohol-insoluble fraction taken.

† This value was obtained by mild hydrolysis (6).

Filtrates were evaporated to 100 ml. under vacuum and then transferred to separatory funnels with ether, and 100 ml. of water were added. The ether solutions were washed with water to give a negative lead test with dithizone and then evaporated to dryness. From Extracts 1 through 5, 2.54 gm. of product were obtained and from Extracts 6 through 10, 1.62 gm. The analytical data are shown in Table I.

Preparation B—In this preparation, a 24.6 gm. sample of the alcohol-insoluble fraction was dissolved in 60 ml. of chloroform saturated with water and added to 500 ml. of methanol. The mixture was warmed to 40–45°, allowed to cool to room temperature, and filtered. The precipitate was redissolved in wet chloroform and reprecipitated five times in the same way. The progress of the fractionation was followed by evaporating and weighing 1 ml. aliquots of the filtrates. No aliquot of the original filtrate was obtained, but aliquots of the filtrates from the five reprecipitations gave values of 8.6, 7.0, 4.3, 3.0, and 1.9 mg., respectively.

This procedure gives a separation similar to that obtained by counter-

current distribution or by the methanol extraction of Preparation A. The methanol solution contains phosphatidyl ethanolamine, inositol-containing phosphatidic acids, and other substances. The methanol-insoluble material, which is discarded, includes that found in the more hexane-soluble phosphoinositide fraction after counter-current distribution or extraction.

To the combined methanol filtrates, 45 ml. of the lead acetate solution were added. Lead salts were removed by filtration, and carbon dioxide was passed into the filtrate. Lead carbonate was filtered and the filtrate was evaporated to 235 ml. under vacuum. The solution was transferred to a separatory funnel with hexane and 250 ml. of water were added. In this experiment the water layer gave a positive dithizone test for lead. This test remained positive after washing the hexane solution four times with water. However, washing twice with 1 per cent acetic acid gave a negative test and three more water washes removed residual acetic acid. The hexane solution was evaporated to dryness under vacuum, leaving 4.89 gm. of a phosphatidyl ethanolamine concentrate whose analysis is shown in Table I.

The purity of this preparation of phosphatidyl ethanolamine was investigated further by determining the amount of unsaponifiable material and the amount and neutral equivalent of the fatty acids present. Samples weighing 171.4 mg. and 162.1 mg. were refluxed for 4 hours with 1 gm. portions of potassium hydroxide in 25 ml. of water. The soap solutions were transferred to separatory funnels and 15 ml. of alcohol were added. They were extracted five times with 25 ml. portions of ether to remove unsaponifiable material. The ether extracts were washed five times with water and the first three washes were returned to the soap solutions. The ether extracts were evaporated and weighed. A value of 12.7 per cent for unsaponifiable material in the phosphatidyl ethanolamine fraction was obtained. The soap solutions were made acid with 2 N H_2SO_4 and extracted three times with ether. The ether extracts were washed to remove sulfuric acid, evaporated to dryness, and weighed. They were then dissolved in neutral alcohol and titrated with 0.05 N NaOH to a phenolphthalein end-point. The phosphatidyl ethanolamine sample contained 62.3 per cent fatty acids with a neutral equivalent of 280.7. Thus a molar ratio of 1.91 fatty acid molecules for each phosphorus atom is obtained. A phosphatidyl ethanolamine whose fatty acids have a neutral equivalent of 280.7 is calculated to contain 1.89 per cent nitrogen and 4.18 per cent phosphorus. Therefore, this sample may be calculated to contain 85.6 per cent phosphatidyl ethanolamine based on its total nitrogen content and 86.5 per cent phosphatidyl ethanolamine based on its phosphorus content. It is seen that phosphatidyl ethanolamine, together with the 12.7 per cent unsaponifiable material, accounts for almost all of the sample.

The unsaponifiable material was found to give a strong test for sterols

and to be insoluble in chloroform or alcohol, but soluble in chloroform-alcohol mixture. This suggests a sterol glycoside. This unsaponifiable material contained 26.3 per cent sugar when analyzed in the same way as described for phosphatides. β-Sitosterol glucoside is calculated to contain 31.2 per cent sugar. Therefore, the principal impurity seems to be sterol glycosides.

As seen in Table I, the nitrogen obtained by periodate oxidation (Burmaster N) is lower than the total nitrogen value. Since Carter *et al.* (8) have reported the presence of a long chain nitrogen base in soy bean phosphatides, a "sphingosine" nitrogen determination was run according to their procedure. A value of 0.07 per cent was obtained which only partially accounts for the difference between total nitrogen and Burmaster N. However, a sample of synthetic α-palmito-α-linoleocephalin, obtained from Dr. W. G. Rose (9), which was calculated to contain 1.96 per cent nitrogen, gave a periodate nitrogen value of 1.55 per cent, indicating that values less than the theoretical may be expected.

DISCUSSION

Upon treatment of soy bean phosphatides with absolute alcohol, phosphatidyl ethanolamine is found in both the soluble and insoluble fractions (1, 10). No satisfactory method has been developed for preparation of phosphatidyl ethanolamine from the soluble fraction, although a partial separation has been obtained by counter-current distribution (10). From the insoluble fraction, on the other hand, phosphatidyl ethanolamine has been prepared in 90 to 95 per cent purity by using lead acetate to precipitate other substances which are present with it in certain fractions obtained by counter-current distribution (1). In this paper, two simplified procedures are presented. Although the phosphatidyl ethanolamine is slightly less pure (85 to 90 per cent), the counter-current distribution step has been eliminated, and the method is suitable for use on a preparative scale. The procedure we prefer is that described under Preparation B. By this method a yield of 19.8 per cent was obtained based upon the weight of the alcohol-insoluble fraction used, while Preparation A gave a total of only 8.4 per cent.

The product seems to be almost free of other phosphatides, and the principal impurity is believed to be sterol glycosides. Attempts to remove sterol glycosides have not been successful. The 2:1 ethanol-chloroform mixture used by Carter *et al.* (8) is not suitable, since the phosphatidyl ethanolamine is also soluble in this mixture.

SUMMARY

A procedure is described for the isolation of phosphatidyl ethanolamine from the alcohol-insoluble fraction of soy bean phosphatides. A concen-

trate containing 85 per cent phosphatidyl ethanolamine is obtained in a yield of 19.8 per cent based on the weight of the alcohol-insoluble fraction.

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ENZYMATIC SYNTHESIS OF γ-GLUTAMYLCYSTEINE*

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Studies on the enzymatic synthesis of glutathione[1] in pigeon liver extracts have demonstrated that γ-glutamylcysteine is the intermediary product and that ATP is required for the synthesis of both bonds in the tripeptide (1). More recent communications from this laboratory (2, 3) have described the purification and the properties of GSH synthetase from both pigeon liver and yeast, enzymes which catalyze the condensation of glycine with γ-glutamylcysteine to form GSH. The present study deals with the partial purification and the characterization of an enzyme, from frozen comminuted hog liver, which catalyzes the formation of γ-glutamylcysteine from L-glutamic acid and L-cysteine. While this investigation was under way, the same reaction was shown to occur in extracts of bean seedlings (4).[2]

EXPERIMENTAL

Materials—Amino acids and peptides were the same preparations that had been used previously (2). Disodium ATP was purchased from the Pabst Brewing Company. Frozen comminuted hog liver was generously made available by Dr. M. Mitz and Dr. S. Yanari of Armour and Company. Purified GSH synthetase from yeast was kindly supplied by Dr. J. E. Snoke of this laboratory.

Methods—During the early stages of enzyme fractionation it was found more convenient to follow Glut.Cyst. synthesis by converting the dipeptide to GSH rather than to assay for Glut.Cyst. directly. Therefore the liver fractions were incubated with L-glutamate, L-cysteine, C^{14}-glycine, excess yeast GSH synthetase, and an ATP regenerating system as described by

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[1] The following abbreviations have been used: GSH, glutathione; Glut.Cyst., γ-glutamylcysteine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenylic acid; P_i, orthophosphate; PGA, 3-phosphoglyceric acid; Tris, tris-(hydroxymethyl)aminomethane.

[2] Attempts to isolate an enzyme with Glut.Cyst. synthetase activity from autolyzed yeast have not been successful in this laboratory.

Snoke *et al.* (2). The incorporation of C^{14}-glycine into GSH was determined (5) and served as a measure of Glut.Cyst. synthesis. Control experiments showed that this is a valid procedure since GSH synthetase possesses no Glut.Cyst. synthetase activity. Moreover, with GSH synthetase in excess, the conversion of Glut.Cyst. to the tripeptide is quantitative. In some experiments C^{14}-glutamate (uniformly labeled) was used instead of C^{14}-glycine. The analytical results were the same within the limits of error of the assay method. In the later stages of purification, after the ATPase activity of the preparations had been sufficiently reduced, the assay for Glut.Cyst. was based on the release of inorganic phosphate from ATP which accompanies formation of the dipeptide from glutamic acid and cysteine. Orthophosphate was determined according to the procedure of Gomori (6) and the concentration of protein measured nephelometrically after precipitation with trichloroacetic acid in 0.1 M $(NH_4)_2SO_4$.

The composition of the reaction mixtures that were used in the two assays is given in Table I.

Enzyme Purification—All operations were carried out at 4°.

Saline Extract—100 gm. of frozen comminuted hog liver were suspended in 227 ml. of 0.04 M $NaHCO_3$ containing 0.8 per cent NaCl. The suspension was adjusted to pH 8.0 with 1 N NaOH, stirred for 90 minutes, and centrifuged at 4000 r.p.m. for 20 minutes. The opaque supernatant solution (200 to 220 ml.) is designated as Fraction A in Table I.

Protamine Sulfate Treatment—The pH of Solution A was adjusted to 6.8 with 0.5 N H_2SO_4 and 40 ml. of a 2 per cent protamine sulfate solution were added. The suspension, after stirring for 30 minutes, was centrifuged at 10,000 r.p.m. to yield 200 ml. of clear red supernatant fluid (Fraction B).

First Ammonium Sulfate Precipitation—Fraction B was adjusted to pH 8.5 with 0.5 N NaOH and brought to 35 per cent saturation with a saturated ammonium sulfate solution, the pH of which had been adjusted to 8.5 with concentrated NH_4OH. The mixture was stirred for 30 minutes, centrifuged, and the precipitate discarded. The supernatant fluid was then brought to 65 per cent saturation with the saturated ammonium sulfate solution and centrifuged after 30 minutes of stirring. The supernatant fluid was discarded, and the precipitate was suspended in a minimal volume of water and dialyzed overnight against cold running tap water. The insoluble material was then removed by centrifugation and the supernatant fluid (Fraction C) was retained.

Second Ammonium Sulfate Precipitation—Fraction C was brought to 40 per cent saturation with saturated ammonium sulfate at pH 5.5. After stirring for 30 minutes, the mixture was centrifuged and the residue was discarded. The ammonium sulfate concentration in the supernatant fluid was then raised to 60 per cent of saturation, the mixture was stirred for 30

minutes, and the precipitated material collected by centrifugation. This precipitate was dissolved in a minimum of water and dialyzed overnight against cold running tap water. The solution was clarified by centrifugation (Fraction D).

TABLE I

Fractionation of Glut.Cyst. Synthetase

Fraction	Glut.Cyst. measured by GSH synthesis*	Glut.Cyst. measured by PO_4 release†	Glut.Cyst. measured by Total protein	Total activity	Glutamine synthetase activity‡	ATPase activity§
			gm.	*μmoles*		
A	0.0018		15	27		
B	0.022		5.2	117		
C	0.081		1.25	101		0.5
D	0.30	0.24	0.30	72	0.1	0.05
	0.35	0.36				
E		0.5	0.10	50	0.00‖	0.02

* Assay systems contained 0.1 M Tris buffer, pH 8.5, 0.1 M KCl, 0.01 M $MgSO_4$, 0.15 M KCN, 0.01 M glutamate, 0.01 M cysteine, 0.015 M C^{14}-glycine, 0.003 M ATP, 0.01 M 3-PGA, 2 mg. of rabbit muscle extract, GSH synthetase, and liver fractions as indicated. The values are expressed as micromoles of GSH formed per mg. of protein at 37° in 60 minutes.

† Measured as micromoles of P_i released per mg. of protein in 60 minutes. Assay systems contained 0.1 M Tris buffer, pH 8.5, 0.01 M $MgSO_4$, 0.00075 M ATP, 0.01 M glutamate, 0.01 M cysteine, and enzyme. Total volume, 2.0 ml.

‡ Measured as micromoles of P_i released per mg. of protein in 60 minutes when cysteine was replaced by 0.01 M $(NH_4)_2SO_4$.

§ Measured as micromoles of P_i released per mg. of protein in 60 minutes when either glutamate or cysteine was absent.

‖ When cysteine was replaced by hydroxylamine in concentrations varying from 0.01 to 1.0 M, only blank values for P_i were observed. Tests for hydroxamic acid by the method of Lipmann and Tuttle (7) were likewise negative. In these experiments the incubation mixtures contained either GSH or KCN to prevent oxidation of SH groups on the enzyme.

Third Ammonium Sulfate Precipitation—Fraction D was adjusted to pH 8.5 with 0.5 M NH_4OH and brought to 45 per cent of saturation with saturated ammonium sulfate solution of the same pH. The suspension was stirred for 30 minutes, centrifuged, and the residue discarded. The ammonium sulfate concentration of the supernatant fluid was then raised to 60 per cent of saturation, the mixture was stirred for 30 minutes, and the precipitated material collected by centrifugation. The precipitate was dissolved in a minimum of water and dialyzed overnight against cold running tap water (Fraction E).

The enzymatic activities of the various fractions of γ-glutamylcysteine

synthetase obtained by the above procedure are given in Table I. It was noted that the *total* activity of Fraction B was 4 times greater than that of Fraction A, while the increase in specific activity was 12-fold. Presumably an inhibitor is being removed, and the actual purification achieved in this step is probably no greater than 3-fold. On this basis it is estimated that the over-all purification of the enzyme was about 60-fold.

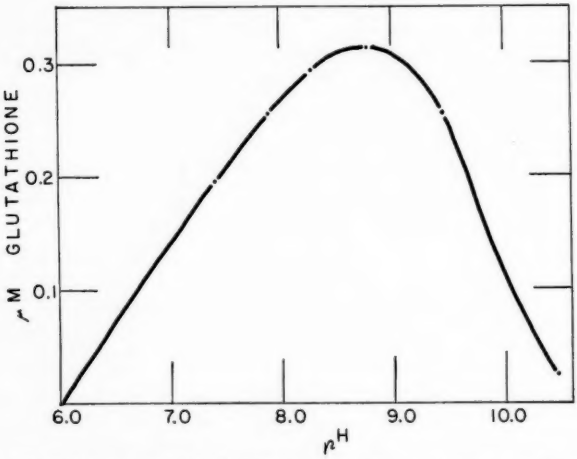

FIG. 1. pH-activity curve of Glut.Cyst. synthetase. Assays were run by incubating glutamate, cysteine, ATP, Mg^{++}, and Glut.Cyst. synthetase as described in Table I, except that the pH was varied as indicated. After incubation for 30 minutes at 37°, the samples were neutralized, boiled, and the precipitated protein removed by centrifugation. An aliquot of the supernatant solution was mixed with C^{14}-glycine and an excess of GSH synthetase. After adjustment of the pH to 8.5, the samples were incubated for 60 minutes at 37°. Standard amounts of Glut.Cyst. were quantitatively converted to GSH under these conditions.

Fraction E was used as the source of enzyme in all the experiments listed in Tables I to V and Fig. 1.

Stability of Enzyme—When Fraction E was stored in the freezing compartment of a refrigerator for 3 months, the enzymatic activity declined to one-half of the original. At pH 5.5 the enzyme is stable in the cold for short periods of time, but rapidly loses activity at room temperature.

pH Optimum—Because of the high ATPase activity exhibited by Fraction E at pH values above 9.3, the dependence of enzyme activity on pH was determined by formation of radioactive GSH, as described in Fig. 1. Optimal Glut.Cyst. synthetase activity was observed between pH 8.3 and 8.8.

RESULTS AND DISCUSSION

The evidence for the identity of the product of Glut.Cyst. synthetase action with glutamylcysteine is 3-fold: (1) incubation of the enzyme with L-glutamate and L-cysteine, in the presence of ATP and magnesium, yields a peptide that has the same R_F value on paper chromatograms as authentic γ-glutamylcysteine; (2) the addition of both glutamate and cysteine is required for the release of inorganic phosphate from ATP; (3) in the presence of glycine and GSH synthetase the product of Glut.Cyst. synthetase action is converted to GSH.

TABLE II

Release of P_i Catalyzed by Glut.Cyst. Synthetase

Components	P_i released
	μmole
Complete	0.61
No cysteine	0.15
" glutamate	0.14
" cysteine, glutamate	0.14
" " " enzyme	0.10
" Mg^{++}	0.12
Plus NaCN (0.015 M)	0.56
" KCl (0.1 M)	0.59
" NaCN (0.015 M), KCl (0.1 M)	0.57

The complete system consisted of 0.1 M Tris buffer, pH 8.5, 0.01 M glutamate, 0.01 M cysteine, 0.01 M $MgSO_4$, 0.00075 M ATP, and 2.4 mg. of Glut.Cyst. synthetase in a total volume of 2.0 ml. Time of incubation, 30 minutes.

For the chromatographic characterization of Glut.Cyst., aliquots of the reaction mixture obtained on incubation of glutamate, cysteine, ATP, and Mg^{++} were used (see Table II for the composition of the medium). The procedure followed was that described earlier for Glut.Cyst. formed in crude pigeon liver extracts (1), with the sole modification that the paper used was Whatman No. 1 instead of Schleicher and Schüll No. 507. Chromatograms were run with solutions containing *N*-ethylmaleimide to promote migration of sulfhydryl compounds, as described by Hanes *et al.* (8). Two ninhydrin-positive spots were obtained, a heavier one which was identified as the imide of cysteine (R_F = 0.43) and a less intense spot which had the same R_F as an authentic sample of γ-glutamylcysteine (R_F = 0.33).

The data in Table I show that the number of micromoles of C^{14}-GSH formed in the assay with radioactive glycine and Glut.Cyst. synthetase is approximately equal to the number of micromoles of P_i that are released

when the reaction mixture contains only glutamate, cysteine, ATP, and Glut.Cyst. synthetase. This result leads to the conclusion that, for each mole of Glut.Cyst. formed, 1 mole of ATP is split with the release of 1 mole of inorganic phosphate, and hence the appearance of P_i is a direct measure of peptide synthesis in this system. Conditions necessary for the formation of P_i from ATP are further described in Table II. That a stoichiometric relation exists between peptide synthesis and ATP utilization is also evident from the relative amounts of P_i released during Glut.Cyst. synthesis on the one hand and those liberated in the total synthesis of the tripeptide on the other. The quantity of P_i that appears as a result of GSH formation in the complete system (containing glycine and both Glut.Cyst. synthetase and GSH synthetase) is twice as great as the quantity of P_i formed when either glycine or GSH synthetase is omitted (Table III). Equal amounts of P_i must therefore be split from ATP during each of the two consecutive steps of GSH synthesis. ATP utilization in the condensation of Glut.Cyst. with glycine has been shown to involve the formation of P_i and ADP in equimolar quantities (3). That orthophosphate and ADP are also the products in the process catalyzed by Glut.-Cyst. synthetase appears likely from the data in Table III. Assays for ADP have not been carried out, since the present preparations of Glut.-Cyst. synthetase appear to contain adenylic kinase which would interfere with the determination of the nucleotide balance. The presence of adenylic kinase is indicated by the finding that P_i is released in significant amounts when ADP replaces ATP in the reaction mixture (Table IV). On the other hand ADP, when added in conjunction with ATP, markedly suppresses the release of inorganic phosphate, presumably by inhibiting the interaction of enzyme and ATP. An inhibitory effect of ADP on Glut.-

TABLE III

Release of Phosphate during Synthesis of Glut.Cyst. and GSH

	P_i released	Δ
	μmole	
Complete	0.78	0.68
No glycine	0.45	0.35
" GSH synthetase	0.45	0.35
" glutamate	0.10	
" cysteine	0.10	

The complete reaction mixture contained 0.01 M glutamate, 0.01 M cysteine, 0.015 M glycine, 0.1 M Tris buffer, pH 8.5, 0.001 M ATP, 0.01 M $MgSO_4$, 0.1 M KCl, 0.015 M KCN, GSH synthetase in excess, and 1.2 mg. of Glut.Cyst. synthetase in a total volume of 2.0 ml. Reaction mixtures were incubated at 37° for 30 minutes.

Cyst. synthesis and hence on P_i liberation from ATP would be consistent with the supposition that ADP is one of the reaction products. Attempts to minimize the effect of adenylic kinase by selective inhibition with fluoride were unsuccessful because Glut.Cyst. synthetase activity was also strongly suppressed by fluoride (Table V).

TABLE IV

Effect of ADP on Glut.Cyst. Synthesis

Experiment No.	ADP	ATP	P_i released
	M	M	μmole
1		7.5×10^{-4}	0.30
2	1.5×10^{-3}		0.11
3	2×10^{-3}	7.5×10^{-4}	0.13
4	5×10^{-4}	7.5×10^{-4}	0.19
5	1×10^{-4}	7.5×10^{-4}	0.27

In Experiment 1, the reaction mixture was that described in Table I. In Experiments 2 to 5, ADP or ADP plus ATP was added in the indicated amounts. Incubations were carried out at 37° for 30 minutes. 1.2 mg. of Glut.Cyst. synthetase were used per experiment.

TABLE V

Effect of Fluoride on Glut.Cyst. Synthesis

Fluoride	P_i released
M	μmole
0	0.28
10^{-5}	0.26
10^{-4}	0.20
10^{-3}	0.10
4×10^{-3}	0.03

The reaction mixture was that described in Table I for P_i release and contained 1.0 mg. of Glut.Cyst. synthetase. Incubation, 30 minutes at 37°.

Since both glutamine and Glut.Cyst. are γ-substituted amides of glutamic acid, it became of interest to ascertain whether the enzyme under study could also catalyze the synthesis of glutamine. When cysteine was substituted by ammonia, liberation of P_i from ATP failed to occur (Table I). The synthesis of the two amides is therefore catalyzed by different enzymes. This conclusion also follows from the observation that cysteine cannot be replaced by hydroxylamine, even in high concentrations. No evidence for hydroxamic acid formation or ATP utilization was obtained. This was true for low concentrations of hydroxylamine (0.01 M) which are

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adequate for the glutamohydroxamic acid formation catalyzed by glutamine synthetase (9), as well as for much higher concentrations of the amine (0.5 to 1.0 M) which are customarily used for the trapping of carboxyl-activated intermediates. It may be recalled that GSH synthetase catalyzes a reaction between Glut.Cyst., ATP, and hydroxylamine with the production of a hydroxamic acid, presumably glutamylcysteinyl hydroxamate, a result which has been taken to mean that it is the carboxyl group in the cysteine moiety of Glut.Cyst. which is activated in the second stage of GSH synthesis (2). Since Glut.Cyst. synthetase fails to catalyze an analogous reaction, it would appear, contrary to expectations, that an activation of the γ-carboxyl group of glutamic acid may not be part of the mechanism by which Glut.Cyst. is synthesized.

Comparison of the two enzymes which are concerned with the synthesis of GSH shows one further notable difference in properties. Potassium ion markedly enhances the action of GSH synthetase (3) but is without effect on the reaction catalyzed by Glut.Cyst. synthetase (Table II).

SUMMARY

An enzyme from hog liver that catalyzes the synthesis of γ-glutamylcysteine from glutamic acid and cysteine has been partially purified. The formation of the dipeptide is dependent upon the presence of Mg^{++} ion and ATP. In the course of the reaction, orthophosphate is released from ATP in stoichiometric amounts.

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BIOCHEMISTRY OF MYOGLOBIN

III. HOMOGENEITY STUDIES WITH CRYSTALLINE BEEF MYOGLOBIN*

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The electrophoretic inhomogeneity of crystalline horse myoglobin was first observed by Theorell and Ehrenberg (1). Repeated recrystallization failed to eliminate the accompanying material and homogeneous myoglobin could only be achieved by the use of preparative electrophoresis. Theorell and de Duve (2) also noticed a colored substance in preparations of human myoglobin which could be separated from the myoglobin by electrophoresis, but the material was not studied further. While studying the acid cleavage of hemoproteins, crystalline horse myoglobin was found to behave as if it were a mixture of three substances (3); more recently, it was found that at pH 8 three colored components could be detected electrophoretically in samples of crystalline horse myoglobin.[1] These three components appear to be the three substances detected during acid cleavage of myoglobin. These studies have now been extended to beef myoglobin in which it has been found that this myoglobin is also inhomogeneous when crystallized by salting out with ammonium sulfate.

EXPERIMENTAL

Preparation of Myoglobin—The isolation procedure used was similar to that of Roche *et al.* (4). However, a brief outline of the fractionation procedure will be presented in order to indicate modifications of their method. This method differs from Theorell's original procedure (5) in that the lead acetate step was eliminated.

Defatted beef skeletal muscle was frozen in dry ice and pulverized. This material was extracted twice with an equal weight of water at pH 6.7. The pH was maintained at this value during the remainder of the isolation. All procedures were carried out at 5° ± 3°. Solid ammonium sulfate was added to 85 per cent saturation and the precipitate discarded. A crude myoglobin precipitate was then obtained by completely saturating the remaining solution with ammonium sulfate. The precipitate was dis-

* Journal Paper No. 103, American Meat Institute Foundation.

[1] Unpublished experiments by U. J. Lewis done in collaboration with H. Theorell and Å. Åkeson, Stockholm, Sweden.

solved in water to give a myoglobin concentration of approximately 20 mg. per ml. and solid ammonium sulfate was added to 80 per cent saturation. A saturated solution of ammonium sulfate was then added dropwise to the point of incipient cloudiness. If after standing 12 hours the precipitate was amorphous, it was removed and dropwise addition of ammonium sulfate solution was continued until another precipitate formed. This process was repeated until crystalline material was obtained. Usually three or four amorphous precipitates formed before the myoglobin crystallized. The amorphous material contained a colorless protein together with some myoglobin. Finally the myoglobin, now in the metmyoglobin form, was twice recrystallized from ammonium sulfate. Photo-

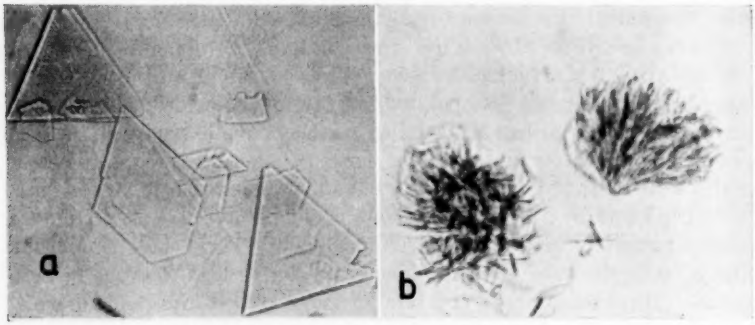

FIG. 1. (*a*), crystals of beef metmyoglobin (× 440) produced by slow crystallization (2 days) from $(NH_4)_2SO_4$ solution; (*b*), the pattern (× 960) obtained when crystallization was rapid (8 hours).

micrographs of the crystals are shown in Fig. 1. Slow crystallization (2 days) produced plates, while needles formed if the process was rapid (8 hours). To our knowledge only the former pattern has been reported (4, 6). The iron content was 0.32 per cent, which is in agreement with the value arrived at by Bowen (7) in his thorough study of horse myoglobin.

Horse myoglobin was prepared by the above method from horse skeletal muscle. Crystallization was much simpler, since the colorless protein that interfered with the crystallization of beef myoglobin was not encountered. The iron content was 0.32 per cent.

Crystallization of pork myoglobin was attempted, but accompanying proteins could not be separated by the ammonium sulfate procedure. At least four other proteins precipitated with the myoglobin in the ammonium sulfate saturation range of 85 to 100 per cent. The contaminating proteins were detected by electrophoresis.

Electrophoretic and Ultracentrifugal Analyses—Electrophoresis was car-

ried out with a Pearson instrument. Photographs were obtained by the Longsworth scanning method with infra-red sensitive film, a red filter, and a tungsten lamp light source. Phosphate buffer, pH 8, $\Gamma/2 = 0.1$, and a migration time of 180 minutes at 20 ma. were employed in all the experiments. Mobilities were calculated from the descending boundaries and were corrected to 0°.

Paper electrophoresis was performed at 5° with Munktell No. 20 paper. Veronal buffer, pH 8.6, $\Gamma/2 = 0.05$, was found to give the best results.

Sedimentation studies were carried out with a Spinco model E analytical ultracentrifuge. Phosphate buffer, pH 8, $\Gamma/2 = 0.1$, together with 1 per cent NaCl, was used in all experiments. The myoglobin and hemoglobin samples were run with a rotor speed of 59,780 r.p.m. The sedimentation constants were corrected to a water basis at 20°.

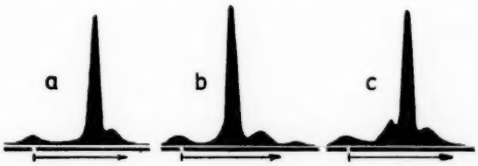

FIG. 2. Tracings of electrophoretic patterns of (*a*) beef myoglobin, (*b*) horse myoglobin, and (*c*) a mixture of beef myoglobin and hemoglobin. A schlieren photograph of the starting boundary is shown for each sample. Ascending boundaries are pictured after 180 minutes of migration at 20 ma.

Acid Cleavage Curves—The acid cleavage procedure involves spectrophotometric determination of the amount of hemin cleaved after addition of a definite amount of myoglobin of known pH to acetone. The time elapsing from the addition of acid to the reading of the optical density was 6 minutes. The amount of hemin cleaved was calculated from the optical density readings and these values were plotted against the pH of the solution. This method has been described previously (3).

RESULTS AND DISCUSSION

Electrophoretic Inhomogeneity—Two distinct components were detected electrophoretically in the crystalline beef myoglobin preparation (Fig. 2, *a*). With the phosphate buffer used the mobility of the main myoglobin boundary was -1.4×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, while the accompanying substance (designated Substance A), which amounted to 15 to 20 per cent of the protein in the mixture, migrated with a mobility of -2×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. The electrophoretic mobilities are summarized in Table I. Numerous recrystallizations did not remove this faster migrating substance. A very small peak (less than 1 per cent) could be seen approxi-

mately half an hour after the current was turned on, but at the end of the experiment (3 hours) the peak had flattened out to such an extent that it did not appear on the photographic plate. The mobility of this small component (designated Substance B) was estimated roughly to be -2.6×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. This substance corresponds to the third component noted in crystalline horse myoglobin preparations (3). An electrophoretic diagram of horse myoglobin is shown in Fig. 2, *b* for comparison. The relative concentrations of Substances A and B were greater in the horse hemoprotein sample, and also the degree of separation of the components was greater in horse myoglobin than in beef myoglobin. Theorell's origi-

TABLE I

Sedimentation Constants and Electrophoretic Mobilities of Hemoglobin and Myoglobin Samples

Solution analyzed	Sedimentation constant (s_{20}) $\times 10^{13}$*	Electrophoretic mobility $\times 10^5$†
	cm. per sec. per unit field of force	*cm.^{2} per sec. per volt*
Myoglobin preparation	1.78	−1.4
80% myoglobin and 20% hemoglobin	1.79	
50% " " 50% "	1.81	
Substance A		−2.0
" B		−2.6
Hemoglobin	4.3	−1.1

* Phosphate buffer, pH 8, $\Gamma/2 = 0.1$, plus 1 per cent NaCl.

† Phosphate buffer, pH 8, $\Gamma/2 = 0.1$.

nal procedure, which makes use of lead acetate for removal of extraneous proteinaceous material, failed to eliminate the faster migrating substances.

Crystalline myoglobin was isolated from four completely different samples of beef skeletal muscle and in all preparations Substance A was present. The concentration of Substance A in these samples fell within the range of 15 to 20 per cent of the protein in the crystalline material. This variation may be similar to the apparently normal variation of myoglobin in beef which was reported in Paper I of this series (8).

Properties of Substance A—It is believed that Substance A is not an artifact produced by denaturation of the myoglobin, since the isolation procedure was kept as mild as possible. The temperature never exceeded $5° \pm 3°$, nor did the pH vary more than 0.5 unit from neutrality before it was readjusted to pH 6.7 and only ammonium sulfate fractionation was employed.

A sample of Substance A was obtained for spectrophotometric study by subjecting a 2 per cent solution of the myoglobin preparation to electro-

phoresis for 10 hours. At the end of that time Substance A had completely separated from the main myoglobin boundary and a portion of this faster migrating material was removed from the cell. This sample of course was mixed with some of Substance B which was present in such low concentration that it could not be seen in the electrophoretic diagram. The light absorption spectrum of Substance A, together with the associated small contamination from Substance B, was very similar to that of metmyoglobin. When treated with pyridine, NaOH, and reducing agent, the reduced pyridine hemochromogen spectrum characteristic of hemin was formed. Substance A, therefore, behaves as if it were a hemoprotein. From acid cleavage data (see below) the heme-protein linkages appear to be different in Substance A and myoglobin.

The physiological significance of Substance A, such as its rôle as a respiratory pigment or enzyme, could not be determined because of the lack of sufficient quantity of the substance. Further study of the chemical and physiological nature of the compound will help elucidate its function relative to other cell constituents.

Attempts were made to obtain larger quantities of Substance A by means of paper and starch electrophoresis. Myoglobin and hemoglobin could be easily separated, but the best resolution of Substance A and myoglobin that could be obtained was the formation of a doublet zone.

The possibility that Substance A might be hemoglobin which had not been completely removed during fractionation was considered. This was found not to be the case, however. Crystalline, electrophoretically homogeneous beef oxyhemoglobin was prepared (9), converted to methemoglobin, and added to a portion of the myoglobin preparation. The concentration of Substance A or B did not increase, while, in fact, the hemoglobin appeared as a third slower moving peak (Fig. 2, *c*). The mobility of the hemoglobin alone or in the presence of myoglobin was -1.1×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, which is considerably less than any of the myoglobin components (Table I).

Ultracentrifugal Studies with Crystalline Myoglobin—The crystalline beef myoglobin was subjected to ultracentrifugal analysis and, although electrophoretically inhomogeneous, the preparation exhibited only one peak during sedimentation (Fig. 3, *a*). The sedimentation constant, s_{20}, for the boundary was 1.78 S (S = 10^{-13} cm. per second per unit field of force). The sedimentation constants are summarized in Table I. The presence of only one peak indicated that either Substance A was of similar molecular weight (assuming similar molecular shape) or that an association complex was formed with the myoglobin which prevented resolution. This problem has not been resolved, but some interesting data on protein association were obtained while attempting to arrive at an answer.

Since Substance A appeared to be a hemoprotein, it was of importance to determine the degree of separation of hemoglobin and myoglobin in the ultracentrifuge. The sedimentation constant of hemoglobin in the buffer system used was first determined and was found to be 4.3 S (Fig. 3, *d*). Then a mixture of 20 per cent hemoglobin and 80 per cent myoglobin (total protein concentration was 5 mg. per ml.) was subjected to ultracentrifugal analysis. Only one boundary with a sedimentation constant of 1.79 S was

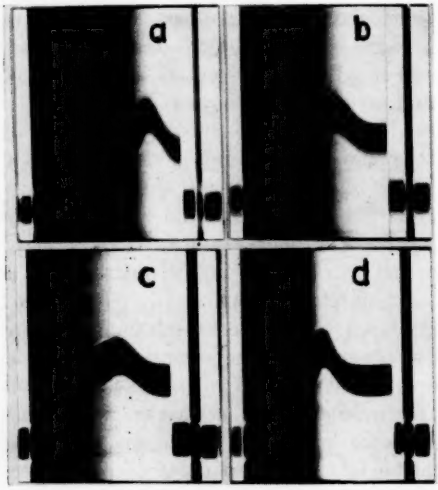

FIG. 3. Velocity sedimentation photographs of (*a*) beef myoglobin, (*b*) a mixture of 80 per cent myoglobin and 20 per cent hemoglobin, (*c*) a mixture of 50 per cent each of myoglobin and hemoglobin, and (*d*) beef hemoglobin. (*a*), (*b*), and (*c*) taken 128 minutes after attaining maximal rotor speed; (*d*) after 96 minutes.

apparent (Fig. 3, *b*). A solution which contained equal amounts of hemoglobin and myoglobin (total protein concentration was again 5 mg. per ml.) was also tested. Again, only one boundary was noted (Fig. 3, *c*). The s_{20} value was 1.81 S. Although two peaks were not found, it can be seen that the sedimentation boundary of hemoglobin or myoglobin alone is much sharper and less diffuse than the boundary of a mixture of the two substances.

It is apparent that, even with a sedimentation constant difference of over 2, myoglobin and hemoglobin cannot be separated by velocity sedimentation in the buffer system employed in these studies. By calculation it was found that, under the conditions used, the two materials should have separated by a distance of nearly 0.6 cm. on the photographic plate.

Therefore, it cannot be assumed that Substance A has a molecular weight similar to that of myoglobin. These results again point out the fact that ultracentrifugal homogeneity alone cannot be considered sufficient evidence for assumption of complete homogeneity. The work of Longsworth (10) with ovalbumin has clearly demonstrated this. Ovalbumin was homogeneous by ultracentrifugation, but more than one component was detected by electrophoresis.

It was surprising to find that the sedimentation constants for the mixtures of hemoglobin and myoglobin were very near the value obtained with the myoglobin preparation alone (Table I). The presence of myoglobin defi-

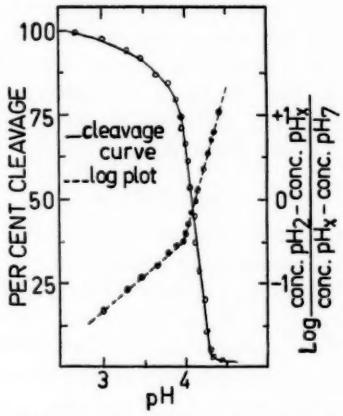

FIG. 4. Acid cleavage of beef metmyoglobin and the logarithmic plot of the data

nitely prevented the hemoglobin from sedimenting at its normal rate. If an association complex were assumed to be formed, it is difficult to understand why the mixture of half myoglobin and half hemoglobin sedimented at a rate nearer to the value for myoglobin rather than to some intermediate value. Although it is only speculation, the data could be interpreted to mean that the hemoglobin dissociated into 4 units of equal size when mixed with myoglobin. Since the molecular weight of hemoglobin is 4 times that of myoglobin, the units would have a molecular weight very close to that of myoglobin and, therefore, would sediment at a rate similar to myoglobin.

Acid Cleavage of Myoglobin—The beef myoglobin was cleaved with HCl at an ionic strength of 0.04 (Fig. 4). Approximately 80 per cent of the hemin was cleaved between pH 4.5 and 4.0. The remaining iron porphyrin was removed by acidifying to pH 2.6. A definite break in the

curve at pH 4.0 was obtained. The break at pH 4.0 is more easily seen if the expression log ((concentration at pH 2) − (concentration at pH x))/((concentration at pH x) − (concentration at pH 7)) is plotted against pH (Fig. 4). The concentration at pH 2 and that at pH 7 are the concentrations of the hemin in acetone at complete and zero splitting, respectively. The concentration at pH x is the concentration of hemin at any point along the curve. From experiments with horse myoglobin and other hemoproteins (3) this indicates the presence of two components. Therefore, the results from the acid cleavage of beef myoglobin are in agreement with the electrophoretic homogeneity data.

The light absorption spectra of samples of the hemin cleaved at pH 4.3 and 2.5 were determined and both agreed with the spectrum of crystalline hemin. This indicates that the two components in the myoglobin preparation possess heme as a prosthetic group. The break in the curve can be interpreted to mean that the hemin of Substance A is more difficult to remove by acid cleavage and, therefore, the heme-protein linkages in this compound are different from those of myoglobin.

SUMMARY

Crystalline beef myoglobin isolated by ammonium sulfate fractionation was found to be electrophoretically inhomogeneous. The accompanying materials have been designated as Substances A and B. Substance A is present to an extent of 15 to 20 per cent, while Substance B amounts to less than 1 per cent of the protein mixture. Neither lead acetate fractionation nor repeated recrystallization from ammonium sulfate removed Substances A and B.

Substance A possessed a light absorption spectrum similar to that of metmyoglobin and, when treated with pyridine and reducing agent, exhibited the reduced pyridine hemochromogen spectrum characteristic of hemin. This behavior is typical of a hemoprotein. Nothing is known of the chemical behavior of Substance B. Substances A and B were shown not to be hemoglobin.

Ultracentrifugal analysis of the myoglobin preparation indicated only one component. However, no conclusions could be drawn as to the molecular weight of Substances A and B, since mixtures of hemoglobin and myoglobin could not be resolved by ultracentrifugation.

We wish to thank Dr. F. W. Putnam of the Department of Biochemistry, University of Chicago, for use of the electrophoresis apparatus and ultracentrifuge. We also wish to acknowledge the assistance of Mr. H. Goldfine in the preparation of the pork myoglobin.

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SIGNIFICANCE OF FERROUS ION AND ASCORBIC ACID IN THE OPERATION OF THE TRICARBOXYLIC ACID CYCLE*

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In 1950, Suda and Takeda (1) found that homogentisicase, an enzyme responsible for the rupture of the benzene ring of homogentisic acid, from rabbit liver requires ferrous ion as a cofactor and hence is strongly inhibited by α,α'-dipyridyl. These findings have been confirmed with rat liver preparations (2–4).

In a succeeding paper (5), it was observed that homogentisicase activity of livers from both α,α'-dipyridyl-injected and ascorbic acid-deficient guinea pigs was markedly lower than that of the control animals and that the lowered activity could be restored by subsequent addition of ferrous ion *in vitro*. Later, Takeda (6) showed that homogentisicase, which had been inactivated by removing ferrous ion, could be reactivated by the addition of ferritin and ascorbic acid. The effect of ascorbic acid was explained by the reduction of the ferric ion of ferritin to the ferrous state and the transfer of the ferrous ion to the homogentisicase. From these observations, it was suggested that ascorbic acid might play an important rôle *in vivo* in the mobilization of iron (6, 7).

On the other hand, Dickman and Cloutier (8, 9) reported that aconitase could be both stabilized and activated by the addition of ferrous ion and reducing agents such as ascorbic acid or cysteine, and effectively inhibited by α,α'-dipyridyl and *o*-phenanthroline. Thus they suggested that ferrous ion acted as a cofactor in the system. Recently Morrison (10, 11) reexamined and confirmed their observations. Further work by Dickman and Speyer (12) verified the intramitochondrial existence of active aconitase.

On the basis of the observations with homogentisicase, one would expect a decrease in aconitase activity in liver following α,α'-dipyridyl treatment or ascorbic acid depletion. Consequently it is possible to assume that in such animals the operation of the tricarboxylic acid cycle will not proceed smoothly, and as a result carbohydrate or fatty acid metabolism will be disturbed, producing ketosis.

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The work reported here was undertaken in order to verify the above supposition *in vivo* and *in vitro*.

EXPERIMENTAL

Young female guinea pigs weighing 300 to 350 gm. were used throughout. The control animals were allowed to consume *ad libitum* the scorbutic diet made from bean curd waste and wheat bran. They also received intraperitoneal injection of a vitamin mixture consisting of 2 mg. of thiamine, 0.05 mg. of riboflavin, 0.05 mg. of pyridoxine, 4 mg. of niacin, 1 mg. of folic acid, and 10 mg. of ascorbic acid per day.

α,α'-Dipyridyl-injected guinea pigs were fed as above and received intraperitoneally 5 mg. per 100 gm. per 24 hours of α,α'-dipyridyl in addition to the vitamin mixture.

Ascorbic acid-deficient guinea pigs were produced by feeding the scorbutic diet mentioned above with the supplement of the vitamin mixture from which ascorbic acid had been omitted. The guinea pigs developed definite scurvy in about 3 weeks, as was evidenced by soreness of the joints and extensive hemorrhage in the fascia of the musculature, particularly of the legs.

All the animals were placed in metabolism cages and 24 hour urine samples were collected under toluene. A ketotic state was produced by giving 1 ml. per 100 gm. per 24 hours of a 25 per cent solution of sodium butyrate (pH 7.0) by stomach tube in three divided doses. Citric and malic acids, both used as a solution of the sodium salt at pH 7.0, were administered simultaneously and isomolecularly with the sodium butyrate in the same volume.

Isolation of Liver Mitochondria—Mitochondria of guinea pig liver were isolated in 0.25 M sucrose by the procedure of Schneider and Hogeboom (13). After being fasted 12 to 24 hours, the animals were killed by decapitation and exsanguinated. The removed livers were weighed, chilled in cracked ice, cut into small pieces with scissors, and homogenized in 9 volumes of cold 0.25 M sucrose in an all-glass Potter-Elvehjem homogenizer. Nuclei and débris were removed by centrifugation at 600 $\times$ g for 10 minutes. The sediment was resuspended in 0.25 M sucrose and recentrifuged at the same speed. The mitochondria were sedimented from the combined supernatant fluids by centrifugation at 8500 $\times$ g for 10 minutes, and washed once with cold 0.25 M sucrose. After resedimentation, the loose fluffy layer above the mitochondria was swirled off. The mitochondria were then taken up for use in cold 0.25 M sucrose.

Determination of Activity of Liver Mitochondria—The mitochondria prepared from 5 gm. of liver were suspended in 6 ml. of 0.25 M sucrose. Citric acid was used as the substrate and the oxygen consumption was measured

manometrically at 38° with oxygen as the gaseous phase. The incubation mixture routinely consisted of the following: 33 μmoles of citrate, 82 μmoles of nicotinamide, 18 μmoles of $MnCl_2$, 10 μmoles of adenosine triphosphate, 0.122 μmole of cytochrome *c*, 0.42 μmole of triphosphopyridine nucleotide, 20 μmoles of phosphate buffer, pH 7.4, and the mitochondrial suspension, in a volume of 1 ml. The volume of the reaction mixture was 3 ml., and 0.2 ml. of 20 per cent KOH was used in the center well.

To avoid the inactivation of various mitochondrial enzymes in the absence of the substrate, the components of the reaction were introduced into manometer vessels at 0°, the gas space was quickly filled with oxygen, and the vessels were equilibrated by shaking in a 38° bath. The taps were closed at the end of 5 minutes and the oxygen uptake recorded for 30 minutes. The oxygen uptake was corrected to include the equilibration period on the basis of the rate recorded during the first 5 minutes (14). The activity (Q_{O_2}) was expressed as oxygen uptake in microliters per mg. of dry weight per 1 hour (corrected for controls without substrate).

Determination of Aconitase Activity—The mitochondria isolated from 5 gm. of liver were suspended in 15 ml. of cold distilled water, followed by freezing and thawing three times to destroy the mitochondrial membrane and the pH adjusted to 7.3 (12). This suspension contained approximately 6 mg. of tissue (dry weight) per ml.

Experiments were carried out anaerobically in evacuated Thunberg tubes at 30°. The reaction was begun by tipping in 1.0 ml. of a solution containing 20 μmoles of *cis*-aconitate (pH 7.3) from the side arm. After 10 minutes in the bath, the reaction was stopped by the addition of 0.5 ml. of 50 per cent trichloroacetic acid. After mixing and cooling in the refrigerator, the solutions were centrifuged and a suitable aliquot was taken for citric acid analysis. The enzyme activity was expressed as an amount of citric acid formed per 100 mg. of dry weight in 10 minutes.

Analytical Procedures—Citric acid was determined by the method of Lardy (15). The color density was measured in a Coleman spectrophotometer at 420 mμ.

Total urine ketones, expressed as acetone, were estimated by a modification of the method of Behre (16, 17). Glucose and other interfering substances were removed by precipitation with copper sulfate and calcium hydroxide (18). The absorption was measured in a Coleman spectrophotometer at 528 mμ.

Results

Urinary Excretion of Ketone Bodies in α,α'-Dipyridyl-Injected Guinea Pigs and Effect of Citrate and Malate upon Ketosis—Beatty and West (19) reported that urinary ketone body excretion increased in the rat fed butyr-

ate, and that the ketonuria was counteracted by the administration of substances related to the tricarboxylic acid cycle. Takeda *et al.* (7) also observed the same phenomenon in guinea pigs.

The control guinea pigs fed butyrate showed a remarkable increase of urinary ketone body excretion from almost zero in the absence of butyrate to an average of 5.2 mg. in a 24 hour period, and the addition of the tricarboxylic acid cycle intermediates, such as citrate or malate, caused a significant decrease of the ketone body excretion to less than 1 mg. per 24 hours. The α,α'-dipyridyl-treated animals likewise showed ketonuria

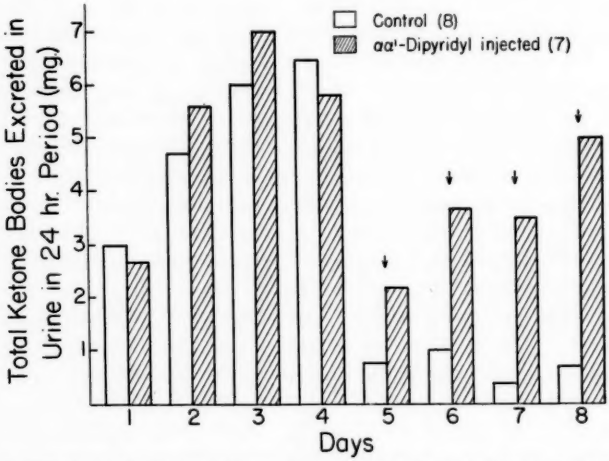

FIG. 1. Effect of citrate on urinary excretion of ketone bodies in α,α'-dipyridyl-injected guinea pigs. The arrows indicate the administration of citrate. The numbers in parentheses represent the number of animals used.

upon the administration of butyrate, but, in contrast, citrate or malate had only a small effect in abolishing the ketonuria. Typical results are shown in Figs. 1 and 2.

Urinary Excretion of Citric Acid in α,α'-Dipyridyl-Injected Guinea Pigs—The administration of α,α'-dipyridyl abolished the effect of the tricarboxylic acid cycle intermediates in reducing ketonuria. If the work *in vitro* on aconitase is applicable to the intact animal, the action of α,α'-dipyridyl might be explained in terms of a reduction in citrate oxidation. It was, therefore, of interest to observe the urinary citric acid excretion in the α,α'-dipyridyl-treated animals.

As shown in Table I, the control guinea pigs excreted an average of 3.4 mg. of citric acid over 24 hours which was not greatly affected by the

addition of butyrate plus citrate or malate. α,α'-Dipyridyl-injected animals excreted an average of 5.1 mg. per 24 hours of citric acid, a value not significantly different from that of the control animals. However, the supplement of butyrate markedly increased the citric acid excretion to 20.7 ± 7.2 mg. per 24 hours and, moreover, the simultaneous administration of citrate or malate with butyrate increased the excretion of citric acid to 47.6 ± 16.5 mg. and 44.2 ± 11.1 mg., respectively.

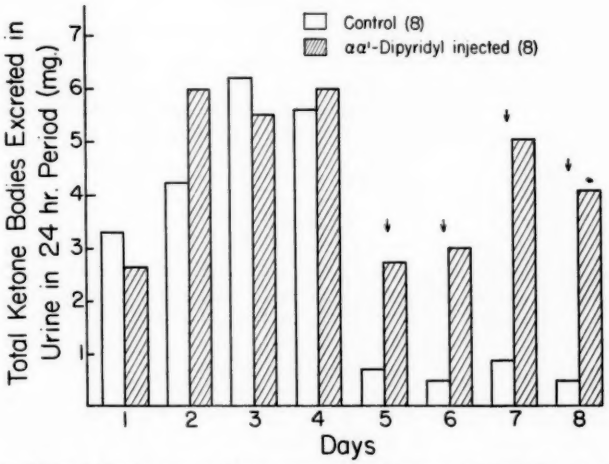

FIG. 2. Effect of malate on urinary excretion of ketone bodies in α,α'-dipyridyl-injected guinea pigs. The arrows indicate the administration of malate. The numbers in parentheses represent the number of animals used.

These results suggest that the major effect of α,α'-dipyridyl is the blocking of the tricarboxylic acid cycle at the stage of citric acid, probably by inhibition of aconitase.

Urinary Excretion of Ketone Bodies and Citric Acid in Ascorbic Acid-Deficient Guinea Pigs—It was suggested that ascorbic acid depletion might cause a decline of the ferrous ion concentration *in vivo* which would subsequently decrease the aconitase activity in liver. If this were the case, one would expect in such animals an increase in urinary excretion of ketone bodies and citric acid, following the administration of butyrate plus citrate or malate.

Experiments were carried out with two groups of ascorbic acid-deficient animals consisting of six guinea pigs each, to which citrate and malate were administered, respectively, together with butyrate. Typical results are given in Fig. 3. Urinary excretion of both ketone bodies and citric

acid markedly increased following the administration of butyrate plus citrate or malate. The increases in citric acid excretion paralleled those of the ketone body excretion.

Inhibition of Citrate Oxidation of Liver Mitochondria by α,α′-Dipyridyl—The inhibitory action of α,α′-dipyridyl on the aconitase of rat liver mitochondria has been already shown (12). The addition of this compound to liver mitochondria from normal guinea pigs revealed 37 and 86 per cent

TABLE I

Urinary Excretion of Citric Acid in α,α′-Dipyridyl-Injected Guinea Pigs

Citric acid excreted in the urine of guinea pigs given 1 ml. per 100 gm. per 24 hours of a 25 per cent neutralized butyric acid solution simultaneously with the test solutions listed. In α,α′-dipyridyl-injected animals, the test solutions were supplemented after 4 days treatment. The values are given in mg.

	Citric acid excreted in urine in 24 hr. period						Averages
Control							
No supplement	2.5	1.6	4.5	3.5	5.6	2.6	3.4 ± 1.5*
Butyrate + citrate	5.8	8.4	11.4	4.0	7.3	5.1	7.0 ± 2.7
" + malate	16.0	7.8	2.4	5.2	9.0	6.5	7.8 ± 4.6
α,α′-Dipyridyl-injected							
No supplement	7.0	6.0	4.2	3.6	2.0	8.0	5.1 ± 2.3
Butyrate	27.0	24.0	21.0	25.1	7.0	20.2	20.7 ± 7.2
" + citrate	30.0	72.1	36.2	61.5	36.5	49.0	47.6 ± 16.5
" + malate	30.0	51.2	43.2	35.0	55.3	50.2	44.2 ± 11.1

* Standard deviation.

inhibitions in citrate oxidation at the final concentrations of 3.3×10^{-4} and 1×10^{-3} M respectively (Table II).

Comparison of Activities of Liver Mitochondria from Normal, α,α′-Dipyridyl-Injected, and Ascorbic Acid-Deficient Guinea Pigs—From the above experiments *in vivo*, it becomes more plausible that α,α′-dipyridyl treatment and ascorbic acid deficiency may cause a decrease in aconitase activity without affecting the condensing enzyme. Further experiments with mitochondrial preparations were performed to confirm this view.

The experimental animals were sacrificed after 5 days of consecutive injections of α,α′-dipyridyl or 21 days of ascorbic acid depletion. As can be seen from Table III, mitochondria from normal animals showed an average Q_{O_2} value of 5.1 in the presence of citrate, while those from α,α′-dipyridyl-treated and ascorbic acid-deficient subjects displayed a marked

decrease in Q_{O_2} to an average value of 0.8 and 0.5, respectively, under the same experimental conditions.

The effect of the time of depletion of ascorbic acid on citrate oxidation was also studied. The results are reported in Table IV. After 5 days deficiency it was decreased by 70 per cent with an average Q_{O_2} value of

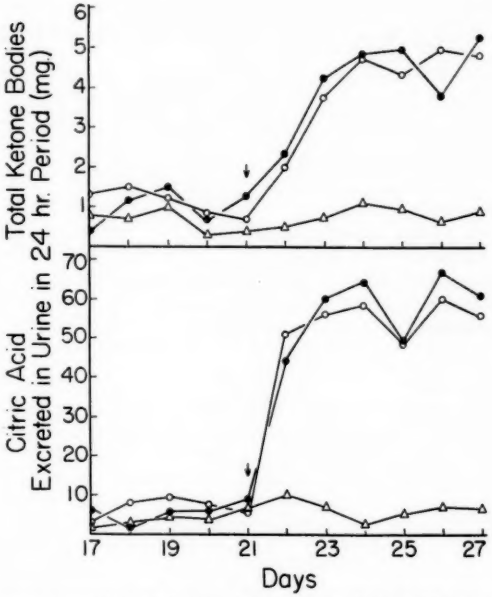

FIG. 3. Urinary excretion of ketone bodies and citric acid in ascorbic acid-deficient guinea pigs. △, normal guinea pigs fed butyrate plus malate; ●, ascorbic acid-deficient guinea pigs given butyrate plus citrate; ○, ascorbic acid-deficient guinea pigs fed butyrate plus malate. The administration of the test substances was started at the time indicated by the arrow and continued for 7 days. The figures on the abscissa represent the number of times guinea pigs were on the scorbutic diet.

1.4. After that, the rate of citrate oxidation decreased progressively with vitamin depletion to a Q_{O_2} value of 0.4 after 20 days deficiency.

From these results, it is concluded that the mitochondrial enzyme systems of guinea pigs involved in the metabolism of citrate are extremely sensitive to ascorbic acid deficiency.

Effect of Ferrous Ion and Ascorbic Acid on Citrate Oxidation of Liver Mitochondria from α,α′-Dipyridyl-Injected and Ascorbic Acid-Deficient Guinea Pigs—If the lowered activity of liver mitochondria from such ani-

mals is due to a decrease in ferrous ion concentration of the liver, it would be expected that the addition of ferrous ion or ascorbic acid might restore the reduced activity.

TABLE II

Inhibition of Citrate Oxidation of Liver Mitochondria by α,α′-Dipyridyl

α,α′-Dipyridyl was added at the final concentrations of 3.3×10^{-4} M and 1×10^{-3} M, respectively, in a volume of 0.2 ml. Otherwise, the conditions were as described in the text.

Experiment		Q_{O_2}*	Inhibition
			per cent
A	Without α,α′-dipyridyl	4.4	
	With α,α′-dipyridyl, 3.3×10^{-4} M†	2.8	37
B	Without α,α′-dipyridyl	5.2	
	With α,α′-dipyridyl, 1×10^{-3} M†	0.6	86

* The values are expressed as oxygen uptake in microliters per mg. of dry weight per 1 hour (corrected for control without substrate).

† Final concentration.

TABLE III

Citrate Oxidation with Liver Mitochondria Isolated from Normal, α,α′-Dipyridyl-Injected, and Ascorbic Acid-Deficient Guinea Pigs

The conditions were as described in the text.

Experiment No.	Q_{O_2}*		
	Normal	α,α′-Dipyridyl-injected	Ascorbic acid-deficient
1	4.6	0.5	0.4
2	5.9	0.8	0.5
3	4.0	1.2	0.4
4	4.9	0.6	0.8
5	4.3	0.8	0.3
6	6.9	1.0	0.4
Averages.....	5.1 ± 1.10	0.8 ± 0.25	0.5 ± 0.18

* The values are expressed as oxygen uptake in microliters per mg. of dry weight per 1 hour (corrected for control without substrate).

The results of such a test are shown in Table V. Contrary to expectations, neither ferrous ion nor ascorbic acid significantly changed the oxygen uptake in the presence of citrate even at the high final concentration of 2×10^{-3} M.

The results obtained suggest the following possible explanations: (1)

Mitochondria may not be permeable to ferrous ion or ascorbic acid. (2) Both α,α'-dipyridyl treatment and ascorbic acid depletion may bring about

TABLE IV

Changes in Rate of Citrate Oxidation with Liver Mitochondria during Development of Scurvy

The conditions were as described in the text.

Experiment No.	Q_{O_2}*					
	1 day-deficient	5 day-deficient	10 day-deficient	15 day-deficient	20 day-deficient	25 day-deficient
1	4.8	1.5	0.6	0.4	0.4	0.2
2	4.2	1.2	0.9	0.6	0.4	0.4
3	4.7	1.6	1.0	0.5	0.5	0.4
4	4.5	1.2	0.6	0.8	0.4	0.5
Averages	4.6 ± 0.27	1.4 ± 0.21	0.8 ± 0.21	0.6 ± 0.17	0.4 ± 0.06	0.4 ± 0.13

* The values are expressed as oxygen uptake in microliters per mg. of dry weight per 1 hour (corrected for control without substrate).

TABLE V

Effect of Fe^{++} and Ascorbic Acid on Citrate Oxidation in Liver Mitochondria from α,α'-Dipyridyl-Injected and Ascorbic Acid-Deficient Guinea Pigs

The test substances were added at the final concentrations listed, in a volume of 0.2 ml. Other experimental conditions were as described in the text.

Addition	Q_{O_2}*		
	α,α'-Dipyridyl-injected	Ascorbic acid-deficient	
None	0.8	1.2	0.4
Fe^{++} (as $FeSO_4$), 3.3×10^{-4} M	0.8		
" 1×10^{-3} M	1.1	1.3	
" 2×10^{-3} "	1.2		0.4
Ascorbic acid (neutralized), 3.3×10^{-4} M	0.8		
" " " 1×10^{-3} M	0.8	1.8	
" " " 2×10^{-3} "	0.8		0.9

* The values are expressed as oxygen uptake in microliters per mg. of dry weight per 1 hour (corrected for control without substrate).

an irreversible decline in the activities of mitochondrial enzymes as a whole, and hence the addition of ferrous ion or ascorbic acid is no longer effective. (3) α,α'-Dipyridyl may inhibit the mitochondrial enzymes related to citrate oxidation, which require metallic ions other than ferrous

ion as a cofactor. However, such inhibitions by this compound have not been observed so far.

Aconitase Activity of Liver Mitochondria from α,α′-Dipyridyl-Injected and Ascorbic Acid-Deficient Guinea Pigs—To avoid a permeability effect, further experiments were carried out with disintegrated mitochondria. Aconitase activity of such preparations from α,α′-dipyridyl-treated guinea pigs was approximately 50 per cent below the normal value (Table VI). The addition of ferrous ion effectively increased the depressed activity.

TABLE VI

Aconitase Activity of Liver Mitochondria from α,α′-Dipyridyl-Injected Guinea Pigs and Effect of Ferrous Ion and Ascorbic Acid*

The amounts of added substances were as follows: enzyme solution, 1.0 ml.; tris(hydroxymethyl)aminomethane buffer (pH 7.3), 20 μmoles; *cis*-aconitate, 20 μmoles; and water to make 3.5 ml. 10 μmoles of Fe^{++} (as $FeSO_4$) and ascorbic acid (neutralized) were added as indicated. Water was substituted for *cis*-aconitate in control tubes. The tubes were incubated at 30° for 10 minutes anaerobically. The values are given in micromoles.

Addition	Citric acid formed			
	Normal		α,α′-Dipyridyl-injected	
	Per tube	Per 100 mg. of dry weight	Per tube	Per 100 mg. of dry weight
cis-Aconitate omitted	0	0	0	0
" added	4.4	69.4	2.2	33.4
+ Fe^{++}	5.6	89.5 (128)†	4.1	62.1 (186)
+ ascorbic acid	4.6	73.0 (105)	2.6	39.4 (118)
+ Fe^{++} + ascorbic acid	5.8	92.0 (132)	5.1	77.6 (233)

* The animals were sacrificed after 5 days of consecutive injections of α,α′-dipyridyl.

† Activation (per cent) in parentheses.

A maximal activation was obtained by supplementing with both ferrous ion and ascorbic acid, while ascorbic acid alone was only slightly effective. In the preparations from normal animals, the addition of ferrous ion or ferrous ion plus ascorbic acid caused a small but definite activation of liver aconitase (Table VI). This may be due to a partial loss of the cofactor, ferrous ion, from the enzyme during the course of preparation.

As in the case of α,α′-dipyridyl treatment, there is a distinct decrease in aconitase activity of liver during the development of scurvy. The results are given in Table VII. The activity was reduced to 70 per cent of the normal value after 5 days depletion and dropped sharply to 33 per cent after 10 days deficiency. Thereafter it decreased progressively with

vitamin depletion to 20 per cent after 25 days deficiency. The addition of ferrous ion or ferrous ion plus ascorbic acid counteracted the lowering of activity, and furthermore the degree of activation was increased gradually with the time of development of scurvy. However, it should be noted that, even though ferrous ion plus ascorbic acid increased the activity, it is still far below the normal value after 25 days depletion.

TABLE VII

Changes in Aconitase Activity of Liver Mitochondria during Development of Scurvy and Activation by Fe^{++} and Ascorbic Acid

The conditions were as described in the text and in Table VI. The values were expressed as an amount of citric acid formed per 100 mg. of dry weight per 10 minutes; the values are given in micromoles.

Days deficient	Citric acid formed			
	No addition	+ Fe^{++}	+ ascorbic acid	+ ascorbic acid + Fe^{++}
Normal	69.4	89.5 (128)*	73.0 (105)	92.0 (132)
	72.6	83.5 (115)	77.5 (107)	88.5 (122)
5	52.1	60.5 (116)	59.1 (113)	71.2 (136)
	48.5	75.6 (156)	56.2 (115)	95.0 (196)
10	25.9	31.4 (125)	26.4 (104)	45.9 (177)
	21.5	31.4 (146)	28.1 (131)	44.6 (208)
15	22.4	34.9 (156)	29.0 (129)	45.0 (200)
	20.0	27.5 (138)	25.0 (125)	36.6 (183)
20	17.8	33.4 (188)	21.6 (116)	43.2 (242)
	16.5	32.1 (195)	20.6 (125)	41.1 (249)
25	12.8	26.4 (206)	15.3 (115)	32.4 (252)
	15.1	30.3 (201)	21.5 (134)	42.4 (281)

* Activation (per cent) in parentheses.

DISCUSSION

From the present studies it is clear that, when guinea pigs are made iron-deficient by α,α'-dipyridyl treatment,[1] or ascorbic acid-deficient, there is a marked decrease in the activity of the mitochondrial enzyme systems in liver.

Although there are many reports on enzymes influenced by ascorbic acid, little is known about its effect on respiratory enzymes. Phillips, Stare, and Elvehjem (20) have reported a decreased indophenol oxidase activity of the liver in both fluorosis and scurvy. According to Harrer

[1] It was observed that the administration of α,α'-dipyridyl caused a severe anemia in rabbits (personal communication from Dr. N. Senda, Osaka University, School of Medicine, Osaka, Japan).

and King (21), cytochrome oxidase showed a moderate decrease in activity in ascorbic acid-deficient heart and skeletal muscle tissue of guinea pigs. The importance of ascorbic acid as a means of protecting sulfhydryl groups of enzymes is well accepted (*cf.* (22)). Harrer and King (21) have reported that, in heart and skeletal muscle of guinea pigs, succinic dehydrogenase, well established as an SH enzyme (23, 24), diminished significantly in its activity upon ascorbic acid depletion. In our laboratory, a 20 per cent lowering of succinic dehydrogenase activity of liver mitochondria was observed in guinea pigs after 20 days on an ascorbic acid-deficient diet (25). The decreases are not as large as those obtained with aconitase. This worker (25) also showed that the dehydrogenase activity of mitochondrial preparations from livers of α,α'-dipyridyl-injected guinea pigs did not differ from that of the normal animals. Therefore, it seems unlikely that, in liver mitochondria from α,α'-dipyridyl-treated and ascorbic acid-deficient guinea pigs, a decrease in succinic dehydrogenase activity is an intrinsic limiting factor of the operation of the tricarboxylic acid cycle.

In α,α'-dipyridyl-injected and ascorbic acid-deficient guinea pigs, the oxidation of butyrate beyond the condensation step of acetyl coenzyme A with oxalacetate appears not to proceed smoothly through the tricarboxylic acid cycle, thereby leading to the accumulation of ketone bodies. This is supported by the observations that urinary citric acid excretion in such animals is significantly increased following the administration of butyrate, and that it is greatly increased by the simultaneous administration of malate or citrate with butyrate. Furthermore, it appears likely that both α,α'-dipyridyl treatment and ascorbic acid depletion have little effect on the condensing enzyme.

From the results of the previous and present experiments, it is likely that ascorbic acid plays a vital rôle in the mobilization of iron *in vivo*. Ascorbic acid deficiency, as in the case of α,α'-dipyridyl treatment, produces a marked decrease in aconitase activity of liver. This seems to be an intrinsic cause for the anomalous excretion of ketone bodies and citric acid in urine.

The rate of decrease in aconitase activity of liver mitochondria parallels the development of scurvy. Although the degree of activation of aconitase by ferrous ion and ascorbic acid increases in inverse proportion to the decline in the enzyme activity, the enhanced activity remains approximately 50 per cent below the normal value in the preparation from 25 day-deficient animals. This fact suggests that ascorbic acid depletion results in a quantitative decrease of the enzyme itself.

From the evidence discussed above, one might make the following assumption: The primary lesion of ascorbic acid deficiency is in the mobiliza-

tion of ferrous ion, causing an inactivation of the enzymes which require ferrous ion as a cofactor, such as aconitase or homogentisicase. Secondly, the inactivation of aconitase induces a disturbance in the operation of the tricarboxylic acid cycle. This, accompanied by a decrease in the activities of other enzymes such as SH enzymes, etc., in turn leads to many metabolic disorders. Thus synthetic reactions, including enzyme formation, are also disturbed, resulting in a quantitative decline in mitochondrial enzymes as a whole.

The permeability effect of the mitochondrial membrane on several substances has been reported (26–28). In our studies, however, the data are insufficient to decide such an effect on ferrous ion and ascorbic acid.

SUMMARY

1. The administration of butyrate to guinea pigs increased urinary ketone body excretion. Citrate or malate was markedly effective in reducing the ketonuria in the control guinea pigs, with only a small effect in α,α'-dipyridyl-injected animals.

2. Urinary citric acid excretion in α,α'-dipyridyl-treated guinea pigs was increased remarkably following the administration of butyrate, and was greatly increased by the simultaneous supplement of citrate or malate with butyrate. The feeding of these acids had no effect on citric acid excretion in the control animals.

3. In ascorbic acid-deficient guinea pigs, urinary excretion of both ketone bodies and citric acid markedly increased following the administration of butyrate plus citrate or malate.

4. Mitochondrial preparations of livers from α,α'-dipyridyl-injected and ascorbic acid-deficient guinea pigs showed a marked decrease in citrate oxidation.

5. Citrate oxidation of liver mitochondria decreased progressively with vitamin depletion.

6. Aconitase activity of liver mitochondria from α,α'-dipyridyl-injected guinea pigs decreased approximately 50 per cent below that from normal animals. The addition of ferrous ion and ascorbic acid restored the depressed activity.

7. Aconitase activity of liver mitochondria was decreased during the development of scurvy. The addition of ferrous ion or ferrous ion plus ascorbic acid partially restored the lowered activity.

8. The significance of ferrous ion and ascorbic acid in the operation of the tricarboxylic acid cycle is discussed.

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RANDOMIZATION OF GLYCERIDE FATTY ACIDS DURING ABSORPTION FROM THE SMALL INTESTINE OF THE RAT

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It has been demonstrated recently that a partial exchange of glyceride fatty acids and free fatty acids takes place by the action of rat pancreatic lipase *in vitro* and *in vivo* (1–3). This exchange which seems to take place largely at the 1,3 positions of the glycerides gives rise to a partial randomization of glyceride fatty acids during digestion in the lumen of the small intestine.

As it is known that, in the rat, glycerides with long chain fatty acids are absorbed to 50 to 75 per cent as glycerides (4, 5), it was of interest to ascertain the extent of randomization that glyceride fatty acids undergo during hydrolysis and absorption. It was thought that such information, besides being of interest *per se*, might define the species of glyceride molecules which are absorbed from the intestine. In this investigation we have characterized the distribution of a labeled acid between free fatty acids and glyceride fatty acids during hydrolysis by rat pancreatic lipase *in vitro* of labeled model glyceride mixtures. With these data in hand it became possible to define the distribution of a labeled fatty acid between the 1,3 and 2 positions of the glyceride from thoracic duct lymph of rats fed the labeled acid dissolved in triglyceride.

EXPERIMENTAL

Hydrolysis of Model Glyceride Mixtures in Vitro Containing Labeled Fatty Acids—Three different glyceride mixtures were used: (1) Olive oil containing 0.2 per cent free palmitic-C^{14} acid. (2) Triglyceride with decanoic-C^{14} acid in the 1,3 positions. The 1-monoglyceride of decanoic-C^{14} acid was prepared from acetone-glycerol and the acid chloride according to standard procedures. Excess of the free labeled acid was removed from the monoglyceride by passing the reaction mixture through a column of the ion exchanger IRA-400 (6, 7). The monoglyceride was then treated with oleyl chloride in excess and the triglyceride isolated by chromatography on a column of silicic acid (8). The labeled triglyceride was then mixed with unlabeled triolein so that the amount of decanoic acid in the triglyceride fatty acids was less than 1 per cent. (3) Triglyceride obtained by transesterification of olive oil with palmitic-C^{14} acid, with sulfuric acid as cata-

lyst (9). This procedure would be expected to result in a random distribution of labeled palmitic acid in the olive oil triglyceride.

These triglyceride mixtures were hydrolyzed by rat bile-pancreatic juice *in vitro* according to procedures previously described (3). About 150 mg. of each mixture were hydrolyzed with 5 ml. of rat bile-pancreatic juice to which was added 1 ml. of 0.1 N hydrochloric acid to bring the pH of the mixture to about 6.4. With this proportion of substrate and enzyme about 65 per cent hydrolysis was obtained in 6 hours at 38° (calculated on

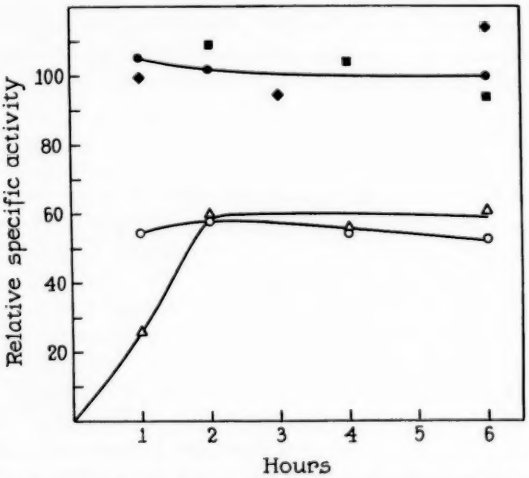

FIG. 1. Specific activities of glyceride fatty acids in per cent of specific activities of free fatty acids during hydrolysis *in vitro* by rat bile-pancreatic juice of (●) olive oil transesterified with palmitic-C^{14} acid; (○) triglyceride with labeled decanoic acid in the 1,3 positions; (△) olive oil containing 0.2 per cent free palmitic-C^{14} acid. Triglyceride obtained from thoracic duct lymph of rats after feeding olive oil containing 0.2 per cent free palmitic-C^{14} acid: ■, Experiment 1; ◆, Experiment 2.

the amount of free fatty acids). The glycerides and free fatty acids of the incubation mixtures at different time intervals were isolated, and the specific activity of their fatty acids was assayed as described earlier (3). The results of these experiments are presented in Fig. 1 in which the specific activities of the glyceride fatty acids are calculated in per cent of that of the free fatty acids at the respective time intervals.

When attention is directed to the experiments in which the mixture of olive oil and free labeled palmitic acid was hydrolyzed, it is apparent that during the course of the hydrolysis free labeled acid has been incorporated into the glyceride. It is, however, also clear that the specific activity of

the glyceride fatty acids only reaches 55 to 60 per cent of that of the corresponding free fatty acids and remains at this level. This result is in accord with that obtained earlier (3) and has been shown to be due to the fact that only the fatty acids in the 1,3 positions of the glyceride are exchanged with the free fatty acids and indicates that the glyceride mixture after an equilibration period contains a constant ratio of fatty acids in the 1,3 and 2 positions.

The course of the curve obtained when synthetic triglyceride containing labeled acids in the 1,3 positions was hydrolyzed was similar to that described above. This result is added confirmation of the above interpretation.

When olive oil transesterified with labeled free palmitic acid was hydrolyzed by rat bile-pancreatic juice *in vitro* according to the same procedure, no differences were found in the specific activities of the glyceride fatty acids and the free fatty acids throughout the course of the reaction. Such a result would be predicted if the labeled fatty acid had been randomly distributed between the 1,3 and 2 positions of the glyceride.

Hydrolysis of Labeled Triglyceride in Vitro from Rat Thoracic Duct Lymph —From the above results it is apparent that information about the distribution of a labeled acid between the 1,3 and 2 positions in a triglyceride can be obtained by following the ratio of the specific activity of the fatty acids of the glyceride mixture to that of the free fatty acids during the course of hydrolysis by rat pancreatic juice *in vitro*. If the labeled fatty acid is predominantly in the 1,3 positions, the specific activity of the glyceride fatty acids during the course of the hydrolysis must be lower than that of the free fatty acids. If the labeled acid is randomly distributed in the triglyceride, the ratio of specific activities of glyceride and free fatty acids will remain at unity throughout the course of the hydrolysis. Finally, if the labeled acid is in the 2 position of the glyceride, the specific activity of the glyceride fatty acids at the beginning of the hydrolysis should be higher than that of the free fatty acids, since initial hydrolysis takes place at the 1,3 positions preferentially. As more and more glyceride molecules are completely hydrolyzed, more and more labeled acid from the 2 position will be freed. Since free labeled acid is then available for exchange with fatty acids in the 1,3 positions of the remaining glyceride, the specific activity of the glyceride fatty acids in per cent of that of the free fatty acids will approach 100 asymptotically. In the following experiments the distribution of a labeled fatty acid in the triglycerides from thoracic duct lymph of rats has been studied after feeding the free labeled acid dissolved in olive oil.

In Experiment 1 olive oil containing 0.2 per cent free palmitic-C^{14} acid was fed to an unanesthetized rat from which thoracic duct lymph was

collected (10) for 24 hours. In Experiment 2 the lymph from five different animals fed the same mixture and treated in the same manner was pooled. The lymph lipides were extracted and the glycerides isolated by chromatography with silicic acid, as described earlier (8). The two lots of lymph triglycerides were then hydrolyzed by rat bile-pancreatic juice *in vitro*, and the specific activities of the glyceride fatty acids and free fatty acids determined as in the above experiments with synthetic glyceride mixtures.

The results shown in Fig. 1 indicate that the free labeled acid fed dissolved in olive oil to rats is randomly distributed between the 1,3 and 2 positions of the glyceride recovered from the lymph of the thoracic duct.

DISCUSSION

It has been shown that the exchange of free fatty acids and glyceride fatty acids during the hydrolysis of glycerides by rat pancreatic juice *in vitro* (1, 2) also takes place during digestion in the lumen of the small intestine of the rat (1, 3). From the results of the experiments *in vitro* it is apparent that this reaction is largely limited to an exchange of fatty acids in the 1,3 positions of the glyceride molecules. The finding in this investigation that a labeled fatty acid fed to rats as the free acid dissolved in olive oil is found randomly distributed between the 1,3 and 2 positions of the lymph glycerides indicates that the partial exchange of the glyceride fatty acids initiated in the lumen of the small intestine by the action of the pancreatic lipase is followed by complete randomization during the passage of glycerides through the cells of the intestinal mucosa.

In another type of experiment Reiser *et al.* (5) provided results indicating that randomization of fatty acids between the glyceride molecules during absorption was not complete. They fed rats a mixture of equal parts of an unlabeled saturated triglyceride and a triglyceride synthesized from glycerol-C^{14} and conjugated linoleic acid. The glycerides recovered from the thoracic duct lymph were subjected to fractionation with alcohol-acetone at 5°, under which conditions triglycerides with two and three saturated fatty acids are relatively insoluble. When the soluble (unsaturated) and insoluble (saturated) glyceride fractions of the lymph glycerides were analyzed, it was found that the "saturated" fraction contained labeled glycerol to a lesser extent than the "unsaturated" fraction. Recombination of free fatty acids with free labeled glycerol has been found not to take place to any significant extent during absorption (4, 5, 11). The formation of "saturated" glycerides containing labeled glycerol was therefore interpreted in the experiments of Reiser *et al.* (5) in terms of an obligatory absorption of glycerol-labeled monoglycerides, followed by resynthesis with free saturated acids in the intestinal mucosa. Since our data demonstrate

that free fatty acids liberated during hydrolysis exchange with glyceride fatty acids in the lumen of the small intestine (1, 2), it is apparent that Reiser's "saturated" glycerol-labeled triglycerides could have been formed also from absorbed di- and triglycerides which had already exchanged part of their fatty acids in the lumen of the intestine. Therefore, data from this type of experiment cannot be used as unequivocal evidence for the species of glyceride absorbed, as has been done by Reiser *et al.* (5) and Reiser (12).

The mechanism by which absorbed glyceride fatty acids are resynthesized during their passage through the intestinal cells is not known. However, the recombination of triglycerides from fatty acids split off in the intestinal lumen can account, at least in part, for the randomization between the 1,3 and 2 positions of the glyceride molecules, since it can be calculated from the results of Reiser *et al.* (5) and of Bernhard *et al.* (4) that from one-fourth to five-sixths of the glyceride fatty acids is split off during absorption.

SUMMARY

Free palmitic-C^{14} acid dissolved in olive oil and fed to rats has been found to be randomly distributed between the 1,3 and 2 positions of the glycerides recovered from the lymph of the thoracic duct. It is concluded that this randomization of glyceride fatty acids, started in the lumen of the small intestine by the action of the pancreatic lipase through partial hydrolysis and exchange, is completed during the passage of the intestinal mucosa to the lymph.

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COMPOUNDS OTHER THAN THREONINE THAT AFFECT FAT DEPOSITION IN THE LIVERS OF RATS FED LOW PROTEIN DIETS*

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The level of fat in the livers of young rats fed a 9 per cent casein-sucrose diet containing choline and the other known lipotropic agents is markedly decreased when additional threonine (0.18 to 0.36 per cent) is included in the diet (1, 2). Harper *et al.* (3) also found that the deposition of liver fat was reduced when the low protein diet was supplemented with a high level (1 to 1.5 per cent) of glycine, serine, glycocyamine, betaine, or choline. The larger amounts of the latter compounds, however, were less effective than threonine.

They also noted that glycine, choline, and betaine further reduced the level of liver fat in rats fed 9 per cent casein diets that contained additional threonine and that combinations of 1.5 per cent glycine with betaine, serine, or choline caused greater reductions than did 1.5 per cent glycine alone. The level of fat in the livers of animals fed a diet containing 2 per cent glycine was lower than that of a group receiving only 1.5 per cent glycine. A mixture of ketogenic amino acids (1 per cent DL-leucine, 1 per cent DL-isoleucine, and 1 per cent L-tyrosine) was also found to reduce deposition of liver fat.

Since the level of choline in the diet was already adequate and since liver fat deposition was not increased when the methionine normally added to the diet was replaced with cystine, it was assumed that neither choline nor the other supplements caused reductions in the level of liver fat by relieving a classical choline deficiency.

The work reported here was undertaken in order to investigate further the effects of glycine and a number of related compounds on the deposition of fat in the livers of rats fed 9 per cent casein-sucrose diets.

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† Stephen Moulton Babcock Fellow.

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Methods

Male weanling rats of the Sprague-Dawley strain, weighing from 40 to 50 gm., were divided into similar groups of six animals each and were maintained in individual cages with raised screen bottoms. The animals were fed *ad libitum*, and each animal was weighed weekly during the experimental period.

The composition of the basal diet was as follows: sucrose 81.6, casein 9.0, DL-methionine 0.3, DL-tryptophan 0.1, corn oil 5.0, Salts 4 (4) 4, and choline chloride 0.15 per cent. Vitamins were included to provide, in mg. per 100 gm. of ration, thiamine hydrochloride 0.5, riboflavin 0.5, niacin 1.0, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, folic acid 0.02, vitamin B_{12} 0.002, and inositol 10.0. 2 drops of halibut liver oil fortified with vitamins E and K (5) were administered orally each week.

The supplements included in the various experimental diets replaced an equal weight of sucrose.

In Experiments 1 to 5 the animals were sacrificed at the end of 2 weeks. In Experiments 6 and 7 the rats were fed the basal diet for 2 weeks after weaning. At the end of this period they were divided into similar groups according to weight and were fed the diets specified in Figs. 1 and 2. Two animals from the control group and five or six from the other groups were sacrificed for the determination of liver fat at 2, 4, and 6 days after the diets had been changed.

The animals were killed by a blow on the head and decapitated. The livers were removed and stored at $-4°$ until the fat determinations were run. Fat was determined by ether extraction of the dried and ground liver (6).

Results

The growth and liver fat results of Experiments 1 to 5 are presented in Table I. The identity of each experiment has been retained in order that each group may be compared with its own control.

The results of Experiment 1 indicated that the reduction of liver fat caused by a mixture of ketogenic amino acids (1 per cent DL-leucine, 1 per cent DL-isoleucine, and 1 per cent L-tyrosine) which had previously been reported was caused by the leucine. In Experiment 2 a supplement of 0.3 per cent DL-leucine was not effective in reducing the level of liver fat, but 1 per cent DL-leucine did cause a slight reduction. In Experiment 3 the level of liver fat of animals receiving 2.7 per cent DL-leucine was even lower than that of the group fed 1.5 per cent glycine, but the rate of gain was also much less. Further work in this laboratory (7) has shown that isoleucine will counteract the growth-depressing effect of high levels of leucine, and, in Experiment 4, although a supplement of 0.5 per cent DL-

TABLE I

*Effect of Various Supplements on Liver Fat Deposition of Rats Fed Low Protein Diet**

Experiment No.	Group No.	Supplement	Rate of gain	Liver fat	
				Dry weight	Wet weight
			gm. per wk.	*per cent*	*per cent*
1	1	None	15.2 ± 1.1	28.9 ± 1.8	9.6 ± 0.7
	2	0.36% DL-threonine	19.3 ± 0.4	14.9 ± 0.7	4.4 ± 0.2
	3	1.0% L-tyrosine	14.9 ± 1.2	29.9 ± 1.5	9.6 ± 0.6
	4	1.0% DL-leucine	15.2 ± 1.4	21.8 ± 2.4	6.8 ± 0.9
	5	1.0% DL-isoleucine	16.6 ± 1.7	27.8 ± 2.3	8.9 ± 0.8
	6	1.0% benzoic acid	15.0 ± 1.0	19.5 ± 2.5	6.0 ± 0.7
	7	1.0% " " + 0.36% DL-threonine	16.9 ± 0.7	11.7 ± 0.6	3.2 ± 0.1
2	8	None	17.7 ± 1.3	31.2 ± 2.9	10.4 ± 1.2
	9	0.75% glycine	16.8 ± 2.1	25.1 ± 2.5	7.7 ± 0.9
	10	1.5% "	14.9 ± 1.2	19.8 ± 3.6	5.8 ± 1.1
	11	3.0% "	14.5 ± 0.5	13.6 ± 0.7	3.6 ± 0.2
	12	1.5% ammonium acetate	18.6 ± 1.0	23.1 ± 1.6	6.9 ± 0.5
	13	1.5% sodium acetate	16.3 ± 1.5	28.2 ± 2.9	8.7 ± 1.2
	14	1.5% " formate	16.2 ± 0.7	26.5 ± 3.5	8.3 ± 1.2
	15	0.3% DL-leucine	19.1 ± 1.5	29.6 ± 2.3	9.4 ± 0.7
	16	1.0% "	16.1 ± 1.7	26.9 ± 2.6	8.4 ± 1.0
	17	1.0% DL-alanine	16.6 ± 1.4	28.7 ± 4.0	9.6 ± 1.9
	18	1.0% DL-phenylalanine	14.7 ± 1.7	26.8 ± 2.6	9.0 ± 1.1
3	19	None	14.1 ± 0.9	30.1 ± 3.1	10.1 ± 1.3
	20	1.5% glycine	14.9 ± 2.3	21.0 ± 1.2	6.2 ± 0.4
	21	3.0% L-glutamic acid	12.9 ± 1.2	21.3 ± 1.5	6.3 ± 0.5
	22	2.7% DL-aspartic "	9.7 ± 0.7	21.6 ± 3.1	6.6 ± 1.2
	23	1.5% ammonium acetate	17.7 ± 1.1	28.2 ± 2.6	9.2 ± 1.0
	24	2.0% DL-serine	14.2 ± 1.6	24.2 ± 1.4	7.2 ± 0.5
	25	2.0% diammonium citrate	17.1 ± 2.0	26.9 ± 1.8	8.7 ± 0.8
	26	2.7% DL-leucine	6.6 ± 0.7	17.5 ± 1.6	5.0 ± 0.5
	27	1.8% DL-alanine	15.4 ± 1.2	27.8 ± 1.5	8.7 ± 0.6
	28	2.8% choline chloride	12.8 ± 0.6	13.2 ± 0.5	3.6 ± 0.2
4	29	None	15.9 ± 0.9	32.2 ± 2.0	10.8 ± 0.8
	30	1.0% L-leucine	11.1 ± 0.8	19.2 ± 1.1	5.9 ± 0.3
	31	1.0% " + 0.5% DL-isoleucine	16.4 ± 0.9	23.7 ± 2.2	7.5 ± 0.8
5	32	None	15.9 ± 1.4	23.4 ± 1.5	7.0 ± 0.5
	33	1.5% glycine	15.4 ± 1.4	19.2 ± 2.0	5.9 ± 0.7
	34	1.0% benzoic acid	14.3 ± 1.1	18.8 ± 1.1	5.6 ± 0.4
	35	1.5% glycine + 1.0% benzoic acid	16.5 ± 1.4	24.4 ± 1.5	7.7 ± 0.5

* The values represent the mean ± the standard error of the mean for six animals.

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isoleucine prevented the growth depression caused by 1 per cent L-leucine, a significant reduction in liver fat deposition still occurred. A comparison of Groups 29, 30, and 31 indicates that the growth depression from the high level of leucine did cause a slight decrease in the level of liver fat.

In Experiment 2 glycine was fed at three levels, and with each increase in the level of glycine a further reduction in liver fat deposition occurred. The level of liver fat of the group receiving the basal diet supplemented with 3 per cent glycine was within the range usually found in animals fed a complete diet.

In Experiment 2 the various supplements were fed at arbitrary levels, while in Experiment 3 they were fed at levels equivalent to 1.5 per cent glycine on a molar basis. Glycine, sodium formate, leucine, aspartic acid, glutamic acid, and choline each caused a reduction in liver fat deposition, while sodium acetate, alanine, and diammonium citrate were without effect. In Experiment 2 the supplement of 1.5 per cent of ammonium acetate caused a reduction in liver fat deposition, but in Experiment 3 when a similar supplement was fed the reduction in liver fat was not significant. The slight reduction in liver fat deposition in the group that received phenylalanine may have been the result of the accompanying growth depression. Aspartic acid also caused a growth depression, which may have contributed to its effect on liver fat deposition.

Since benzoic acid is known to be combined with glycine and excreted as hippuric acid, the effect of this compound on liver fat deposition was tested in Experiment 1. Benzoic acid, fed at a level of 1 per cent of the diet, caused a reduction in the deposition of liver fat. It also caused a reduction when fed with 0.36 per cent DL-threonine. The reduction in liver fat deposition caused by feeding 1 per cent benzoic acid was of the same magnitude as that produced by a supplement of 1.5 per cent glycine. It was impossible to study the effect of higher levels of benzoic acid, because larger supplements caused a depression in growth and in some cases death. In Experiment 5, when 1.5 per cent glycine was fed with 1 per cent benzoic acid, there was no effect on liver fat deposition, yet, when these supplements were fed separately in the same experiment, each caused a reduction in the level of liver fat.

The results of Experiments 6 and 7, which were designed to study the rates at which the different supplements caused fat to be removed from the liver, are presented in Figs. 1 and 2. In these experiments the animals were fed the basal diet for 2 weeks before the supplements were added. The reduction in the amount of liver fat which was caused by each supplement was measured after 2, 4, and 6 days.

In Experiment 6 (Fig. 1) the amount of fat in the livers of the rats receiving the basal diet supplemented with threonine decreased gradually;

however, after 6 days the level of liver fat was still not so low as is usually found in rats fed this diet from the time of weaning. . At the end of 2 days glycine had exerted its maximal effect, and no further decrease in the amount of fat in the liver occurred in the next 4 days. The level of liver fat of the group fed the basal diet supplemented with both threonine and glycine was lower than that of either of the other groups at each point.

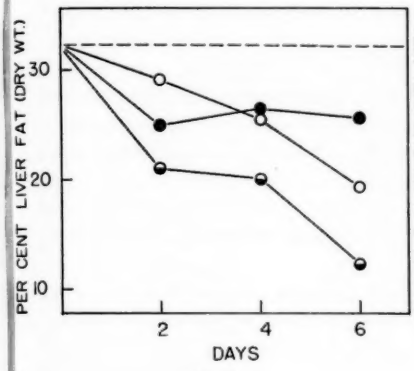

FIG. 1

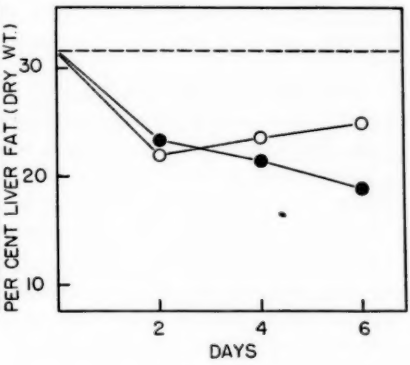

FIG. 2

FIG. 1. Experiment 6, level of liver fat plotted against the time in days after the supplements were first included in the diets. The rats were fed the basal diet for 2 weeks before they received the supplements. ○, 0.36 per cent DL-threonine; ●, 1.5 per cent glycine; ◒, 0.36 per cent DL-threonine and 1.5 per cent glycine. Each point represents the average of five animals. The broken line represents the average of six animals which were fed the basal diet. Two animals were killed at 2, 4, and 6 days.

FIG. 2. Experiment 7, level of liver fat plotted against the time in days after the supplements were first included in the diets. The rats were fed the basal diet for 2 weeks before they received the supplements. ●, 1.5 per cent glycine; ○, 3.0 per cent L-glutamic acid. Each point represents the average of six animals. The broken line represents the average of six animals which were fed the basal diet. Two animals were killed at 2, 4, and 6 days.

Therefore the effects of glycine and threonine appeared to be additive when they were fed together. Experiments with each of these groups have been repeated in two other tests which are not reported in Table I, and similar results were obtained. The rate of growth of the group receiving threonine alone was 4.0 gm. per day during the 6 day experimental period. The group receiving threonine and glycine grew 4.8 gm. per day, while the groups receiving the basal diet or the basal diet plus glycine grew only 2.5 gm. per day.

The effects of glycine and glutamic acid in equimolar amounts were compared in Experiment 7 (Fig. 2). The groups that received rations supplemented with 1.5 per cent glycine or 3.0 per cent glutamic acid showed similar decreases in the level of liver fat in 2 days. Although in the group receiving glycine there was a slight decrease in liver fat and in the group receiving glutamic acid an increase at 4 and 6 days, the differences are of doubtful significance in view of the results with glycine presented in Fig. 1. These groups grew at similar rates throughout the experimental period.

DISCUSSION

Previous work has shown glycine, serine, glycocyamine, betaine, choline, and threonine to be effective in reducing the level of liver fat in rats receiving the basal diet. The present work has added leucine, glutamic acid, aspartic acid, and benzoic acid to the list of effective compounds. Glycine, serine, betaine, glycocyamine, and choline are related by structure and metabolism, but the five other compounds which have been found to be active are not so closely related.

Equimolar levels of glycine, serine, and glutamic acid caused reductions of similar magnitude in liver fat, while an equivalent supplement of choline produced a greater response. Benzoic acid also was more active than glycine, since in animals receiving 1 per cent benzoic acid the level of liver fat was similar to that of animals receiving 1.5 per cent glycine, although this level of glycine is 2.4 times that of the benzoic acid on a molar basis.

Among the compounds which had no apparent effect on liver fat deposition were ammonium acetate, ammonium citrate, and urea. This eliminates the possibility that the effective supplements owe their activity to the non-specific nitrogen they supply.

If a lack of endogenous glycine causes fat to accumulate in the liver, the addition of benzoic acid to the diet should increase the level of liver fat by causing the excretion of glycine as hippuric acid. Since benzoic acid caused a decrease in liver fat deposition, the cause of the liver fat accumulation cannot be the inability of the rat to synthesize sufficient glycine.

The results of the curative trials, in which the maximal effect of glycine occurred more rapidly but was of lesser magnitude than that of threonine, together with the difference in the effective amounts of these two compounds both on a molar and on a weight basis, indicate that their modes of action are different. Because threonine is one of the essential amino acids limiting growth on the basal diet, the slower rate found with threonine is consistent with the suggestion that threonine is needed for the synthesis of an enzyme (1, 2) or hormone involved in liver metabolism, since protein regeneration might well be relatively slow in rats receiving low protein

diets. Glycine, which is a non-essential amino acid, must cause the reduction of liver fat by some rapid process which will circumvent the effect of the limiting protein. Since the rate and extent of liver fat removal caused by glutamic acid are similar to those caused by an equimolar amount of glycine, their modes of action may be similar.

Artom (8) has studied fat oxidation in the livers of rats fed a low protein, choline-deficient diet. When 0.4 per cent choline was added to the diet, fat oxidation, as measured by isotopically labeled CO_2 liberated by liver slices, homogenates, and preparations of liver mitochondria, was greatly enhanced. The injection of massive doses of choline shortly before the animals were killed caused an even greater increase in fatty acid oxidation. The livers of the animals which had received choline in the diet also showed marked increases in fatty acid oxidation when injections of choline were given shortly before the animals were killed. The addition of choline, betaine aldehyde, betaine, or phosphorylcholine to the liver preparations did not increase fatty acid oxidation. A similar study of the compounds that have been found to affect the deposition of liver fat might help to explain their mode of action. Preliminary work in this laboratory indicates that a supplement of 3 per cent glycine to the basal diet does not cause an increase in the endogenous respiration of liver homogenate from the animals.

Although the metabolic pathways of all of the compounds which are active in reducing liver fat are not completely known, it is of interest to note that glutamic acid can be converted into α-ketoglutaric acid, which is an intermediate in the tricarboxylic acid cycle. Large amounts of this acid produced in the liver from glutamic acid might increase the oxidation of fat by this system. Although glycine is not known to be converted directly into a tricarboxylic acid cycle intermediate, aspartic acid, which yields oxalacetic acid, is also effective in reducing liver fat deposition.

SUMMARY

The effects of various supplements on the deposition of liver fat in rats fed a low protein diet containing choline and methionine were studied. Leucine, glutamic acid, aspartic acid, and benzoic acid were found to be effective in preventing excessive deposition of liver fat.

Supplements of glycine, serine, and glutamic acid, equivalent to 1.5 per cent glycine on a molar basis, caused reductions in the level of liver fat which were of similar magnitude, while choline fed at a similar level was more effective.

Dietary supplements of 1.5 per cent of glycine and 3.0 per cent of glutamic acid produced their maximal reduction of liver fat in 2 days, while the effect of 0.36 per cent of DL-threonine did not appear to be complete

even after 6 days. The effect of threonine on fat deposition in the liver was thus considered to be different from that of the other two compounds. Possible explanations for these differences are discussed.

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ACTIVITY OF LIVER GLUTAMINASES IN VITAMIN B_6-DEFICIENT RATS*

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In this laboratory, a lowered level of plasma glutamine has been found consistently in rats deprived of vitamin B_6 and given deoxypyridoxine (1–4), although not in rats simply deprived of vitamin B_6 for 20 days (5). Results following intraperitoneal administration of glutamic acid to vitamin B_6-deficient rats suggested a decreased formation, or increased utilization, of glutamine (2). Since plasma glutamic acid was generally higher in vitamin B_6-deficient rats than in pair-fed controls (1–4), renal phosphate-activated glutaminase I activities were investigated (6). After 35 days of vitamin restriction, the renal glutaminase activity of vitamin B_6-deficient rats was significantly lowered compared with pair-fed controls. With dietary provision of deoxypyridoxine this lowering was elicited after only 20 to 22 days of vitamin B_6 deprivation. Further, when pyridoxal phosphate was added to the kidney homogenates of vitamin B_6-deficient rats, renal phosphate-activated glutaminase activities were restored to normal (6).

In rat liver two separate glutaminase enzymes have been demonstrated: glutaminase I (phosphate-activated) and glutaminase II (pyruvate-activated). Glutaminase I occurs in numerous animal tissues, both normal and neoplastic (7, 8), whereas glutaminase II is apparently restricted to liver tissue and liver tumors (7, 9). In view of the above findings on glutamine and renal phosphate-activated glutaminase I in vitamin B_6-deficient rats, it was felt advisable to investigate activities of liver glutaminases I and II in deficient animals. The results of these investigations are reported in this communication.

Methods

Albino rats of the Wistar strain and of both sexes were employed. All animals were housed in individual, screen-bottomed cages, and drinking water was provided *ad libitum*. The 20 per cent casein, 20 per cent corn oil, vitamin B_6-free basal diet described previously (1) was fed. Vitamin B_6, when supplied, was added to the food as pyridoxine hydrochloride at a

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level of 50 γ per rat per day. In all cases, pyridoxine-fed controls were pair-fed with their comparable vitamin B_6-deprived groups to eliminate differences in experimental results due to differences in the amount of food consumed. Following the indicated period of experimental feeding, the animals were fasted for 20 hours and killed by stunning and decapitation. The livers were quickly removed, rinsed in saline, blotted, and an aliquot was weighed for determination of enzyme activity.

The activity of phosphate-activated glutaminase I of liver tissue was estimated by a modification of the procedure of Archibald (10) as applied to renal tissue by White and Rolf (11). A 5 per cent liver homogenate was prepared in phosphate-cyanide buffer (pH 8.1) with a Potter homogenizing tube immersed in ice water. Each reaction tube contained 1 ml. of tissue homogenate, 1 ml. of L-glutamine (20.8 μmoles), and 2 ml. of 0.2 M phosphate buffer, all at pH 8.1, the optimal pH for liver glutaminase I (12). For each rat a tissue blank was used containing 1 ml. of tissue homogenate, 1 ml. of water, and 2 ml. of 0.2 M phosphate buffer. In each run blanks containing 1 ml. of phosphate-cyanide buffer, 2 ml. of 0.2 M phosphate buffer, and 1 ml. of water or glutamine were used to correct for non-enzymatic hydrolysis of glutamine during incubation and nesslerization. Incubation was carried out for 30 minutes at 37°. It might be thought that pyruvate-activated glutaminase II of liver tissue would interfere in this procedure. In this respect Errera (13) reported that cyanide inhibits purified liver glutaminase II. In this laboratory it was found that addition of 31.8 μmoles of sodium pyruvate to the reaction mixture stimulated ammonia formation in this procedure when cyanide was absent. In the presence of cyanide in the buffer as described, ammonia in tissue blanks was decreased and no stimulation of ammonia formation was noted on addition of pyruvate to the reaction mixture.

The activities of pyruvate-activated glutaminase II were estimated by the method described by Beaton (14) with a 4 per cent aqueous liver extract. Adequate tissue controls were used for each rat as described in the method (14). Each reaction tube contained Veronal buffer (pH 7.7), 31.8 μmoles of sodium pyruvate (pH 7.7), 40 mg. of tissue, and 20.4 μmoles of glutamine or water, incubated 2 hours at 37°.

Pyridoxal phosphate was added to an aliquot of the homogenates of the livers of each animal in the manner described for renal glutaminase (6). The diammonium salt of pyridoxal-5-phosphate was obtained from the California Foundation for Biochemical Research and was added to an aliquot of each homogenate at a level of 33 γ per 100 mg. of tissue. Calculated as pure pyridoxal phosphate, this level (20 γ per 100 mg.) is approximately 2 times that found effective for certain transaminase systems (15). Addition of pyridoxal phosphate to the aliquots of homogenates was made

1 hour prior to the assay of glutaminase I and II activities, and the homogenates with and without added pyridoxal phosphate were stored in a cold room at 4°. Tissue blank corrections in the glutaminase assays were made for the addition of pyridoxal phosphate.

In one experiment free glutamine levels were determined in kidney and liver tissue. Individual 5 per cent homogenates of these tissues were made in cold 5 per cent trichloroacetic acid with a Potter homogenizing tube immersed in ice water. The homogenates were filtered, and free glutamine was determined in aliquots of the filtrates by the colorimetric procedure of Prescott and Waelsch (16) as modified by Bessman *et al.* (17).

In all cases, a *t* test was applied to ascertain the significance of differences between mean values of deprived and control animals.

EXPERIMENTAL

Activity of Liver Phosphate-Activated Glutaminase I in Vitamin B_6-Deficient Rats—Eighteen rats were divided into two groups comparable with respect to number, sex distribution, and an initial average body weight of 100 gm. One group was deprived of vitamin B_6; the remaining group received pyridoxine and pair-fed with the deprived group. After 28 to 30 days of experimental feeding the animals were fasted, killed, and phosphate-activated glutaminase I activities measured in liver homogenates with and without added pyridoxal phosphate. Since the number of animals which could be assayed in 1 day was a limiting factor, care was taken to select rats from each group and each sex each day of the 3 day period of analyses to prevent biasing of results. During the feeding period the average daily food intake of both groups was 13.0 gm. per rat, and average gains in body weight were 61 and 112 gm. for the deficient and control groups respectively. The results of glutaminase I assays are reported in Table I.

Activity of Liver Pyruvate-Activated Glutaminase II in Vitamin B_6-Deficient Rats—Eighteen rats were divided into two groups comparable with respect to number, sex distribution, and an initial average body weight of 107 gm. One group was deprived of vitamin B_6; the remaining group was given pyridoxine and pair-fed with the deprived group. After 35 to 37 days of experimental feeding, the animals were fasted, killed, and pyruvate-activated glutaminase II activities measured in liver homogenates with and without added pyridoxal phosphate. Again, care was taken to select animals of each sex from each group on each day of the 3 day period of analyses. In this experiment, free glutamine concentrations were estimated in both liver and kidney homogenates prepared in trichloroacetic acid. The average daily food consumption of both groups was 12.6 gm. per rat, and average body weight gains were 45 and 105 gm. for the deficient and con-

trol groups respectively. The results of glutaminase II and free glutamine determinations are presented in Table II.

Dialysis of Liver Extract and Glutaminase II Activity—Seven stock, chow-fed young rats were sacrificed by stunning and decapitation, and a 4 per

TABLE I

Activity of Phosphate-Activated Glutaminase I of Liver Tissue from Vitamin B_6-Deficient and Pair-Fed Control Rats (Nine Rats per Group)

Group	Pyridoxal phosphate*	Liver glutaminase I activity†	
		Range	Mean ± s.d.
Deficient	−	32.7–46.8	42.9 ± 5.3
	+	9.9–42.9	32.2 ± 9.5
Control	−	21.1–41.3	31.6 ± 6.8
	+	8.5–39.5	24.0 ± 10.1

* 20 γ per 100 mg. of liver tissue (wet weight). Homogenates with and without added pyridoxal phosphate were stored at 4° for 1 hour prior to assay.

† Expressed as micromoles of glutamine hydrolyzed by 100 mg. of liver tissue (dry weight) in 1 hour.

TABLE II

Activity of Pyruvate-Activated Glutaminase II of Liver Tissue and Free Glutamine Concentrations of Liver and Kidney Tissue from Vitamin B_6-Deficient and Pair-Fed Control Rats (Nine Rats per Group)

Group	Pyridoxal phosphate*	Liver glutaminase II activity†		Free glutamine, mg. per 100 gm.	
		Range	Mean ± s.d.	Liver	Kidney
Deficient	−	0 − 3.79	1.19 ± 1.57	150 ± 33	76 ± 17
	+	0 − 4.82	2.16 ± 1.65		
Control	−	5.23–11.40	7.23 ± 2.31	129 ± 22	57 ± 20
	+	3.78–11.60	6.97 ± 2.50		

* 20 γ per 100 mg. of liver tissue (wet weight). Homogenates with and without added pyridoxal phosphate were stored at 4° for 1 hour prior to assay.

† Expressed as micromoles of glutamine hydrolyzed by 100 mg. of liver tissue (dry weight) in 1 hour.

cent aqueous extract of liver was prepared and dialyzed against cold running water at 14° for 4 hours. At the end of this time pyruvate-activated glutaminase II activity was measured in the dialysate and compared with the activity of an aliquot of the 4 per cent aqueous extract stored in a test-tube in cold running water at 14° for 4 hours. Pyridoxal phosphate was added to an aliquot of each dialysate at a level of 100 γ per 40 mg. of tissue immediately prior to assay of activity. Dialysis of the liver extract

resulted in decreases of glutaminase II activity in all extracts, ranging from 30 to 100 per cent. On addition of pyridoxal phosphate to the dialyzed extracts, erratic stimulation of glutaminase II activity resulted in five of the extracts, ranging from 30 to 92 per cent of the stored extract's activity. In two of the dialyzed extracts, added pyridoxal phosphate was without effect.

RESULTS AND DISCUSSION

It should be emphasized that any differences between the deficient and control groups in each experiment cannot be attributed to differences in the amount of food consumed, since a paired feeding technique was employed. Further, 4 or 5 weeks of vitamin B_6 restriction with the basal diet employed here are adequate for demonstrating abnormalities *in vivo* and *in vitro*, including a lower transaminase activity (18).

As shown in Table I, an elevation in phosphate-activated glutaminase I activity was evident in vitamin B_6-deficient rats ($t = 3.61$; significant at the 1 per cent level). Addition of pyridoxal phosphate resulted in a lowering of this enzyme's activity in both groups, significant at the 2 per cent level in vitamin B_6-deficient homogenates ($t = 2.79$), but not significant in control homogenates owing to the large variation ($t = 1.77$). In a second duplicate experiment, essentially the same results were obtained.

As shown in Table II, vitamin B_6 deficiency resulted in a lowering of liver pyruvate-activated glutaminase II activity ($t = 6.43$; significant at the 1 per cent level). Indeed, no activity could be detected in five of the nine livers studied from deficient rats. Under the conditions described, addition of pyridoxal phosphate to the liver extracts of vitamin B_6-deficient rats resulted in an insignificant mean increase in glutaminase II activity. No stimulation of activity was noted in three of these liver extracts. Added pyridoxal phosphate was without effect on glutaminase II activities of control liver extracts. These findings suggest that pyridoxal phosphate has a relatively small effect on the activity of liver glutaminase II under these conditions, although this effect may be more apparent in dialyzed liver extracts. Meister *et al.* (19–21) have recently demonstrated an α-keto acid-catalyzed glutamine- (or asparagine-) transamidase-deamidase system in rat liver. When a reaction system similar to that employed here was used for measuring liver glutaminase II activity, no alteration was found in the formation of ammonia from glutamine in vitamin B_6-deficient rat livers (22). The experimental conditions of Meister *et al.* (22) differed considerably from those used here. Calculated on the same basis, pyruvate-activated glutaminase II activities reported by us are greater than those reported by Meister *et al.* (22) and are comparable to activities found in chow-fed stock rats (14). Under our conditions glu-

taminase I activity in liver tissue of control rats was considerably greater than glutaminase II activity.

Free glutamine concentrations of liver and kidney tissue (Table II) were higher in these tissues of vitamin B_6-deficient rats than in their pair-fed controls. However, variation was such that statistical significance of difference between means was not attained. This finding suggests that the lowering in liver pyruvate-activated glutaminase II and in renal glutaminase I activities (6) in vitamin B_6-deficient rats is probably not an adaptation to a decrease in available substrate.

In view of the numerous implications of vitamin B_6 in amino acid metabolism, it is perhaps not surprising that vitamin B_6 appears to affect glutamine metabolism. The earlier reports of low levels of plasma glutamine (1–4) and lowered renal glutaminase I activity (6) and the present observations on liver glutaminase I and glutaminase II activities and tissue levels of free glutamine in vitamin B_6-deficient rats indicate that vitamin B_6 may be involved in the metabolism of glutamine.

SUMMARY

Activities of phosphate-activated glutaminase I and pyruvate-activated glutaminase II have been measured in livers of vitamin B_6-deficient and pair-fed control rats. In vitamin B_6-deficient rats, liver glutaminase I activity was significantly higher and liver glutaminase II activity was significantly lower than in pair-fed controls. Addition of pyridoxal phosphate to liver extracts of vitamin B_6-deficient rats resulted in a restoration of only a very small part of the activity of glutaminase II. An erratic but relatively positive stimulation of glutaminase II activity by added pyridoxal phosphate was noted in dialyzed liver extracts. Data on liver and kidney free glutamine concentrations indicate that decreases in enzyme activities are probably not attributable to decreases in available substrate.

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THE EFFICIENCY OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM DIABETIC RATS*

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Considerable evidence has accumulated which indicates that insulin plays an important rôle in phosphate metabolism (1, 2). A number of workers have postulated that insulin has as its primary function the regulation of the efficiency of oxidative phosphorylation. In attempts to test this hypothesis, Goranson and Erulkar (3) observed that tissues from alloxan-diabetic rats appear to fix phosphate less efficiently than do those from normal animals, and Polis *et al.* (4) reported that insulin added *in vitro* increases phosphate fixation in aged preparations from normal rats. When P:O ratios[1] are computed from their data, however, it is revealed that the former workers (3) obtained ratios between 0.03 and 0.2 and the latter (4) between 0.2 and 1.6. Since presently attainable P:O ratios are 2 to 100 times greater than these, the effects that were observed are difficult to interpret and have questionable relevance to the problem of insulin action. Judah and Williams-Ashman (5) were unable to detect significant alterations in P:O ratios when insulin was added *in vitro* to "cyclophorase" preparations from normal animals.

Methods are now available for the isolation of mitochondria of high phosphorylating ability (6). By the use of such mitochondria from liver, the P:O ratios for certain one- and two-step oxidative reactions have been measured for both normal and alloxan-diabetic rats. No differences which could be ascribed to insulin deficiency were detected.

EXPERIMENTAL

Production of Alloxan Diabetes—40 mg. of freshly dissolved alloxan per kilo of body weight were injected intravenously into adult (175 to 200 gm.)

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[1] Moles of inorganic phosphate removed from the medium divided by gm. atoms of oxygen consumed.

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albino rats, following a 48 hour fast as recommended by Kass and Waisbren (7). Studies were performed on two groups of animals; in the first series ("short term" diabetics), animals were sacrificed 6 to 10 days after alloxan injection, and in the second ("long term" diabetics) 3 weeks to 6 months after treatment with alloxan. The development of diabetes was noted by the occurrence of glucosuria, polyuria, polyphagia, polydipsia, and weight loss. Bilateral cataracts developed in many of the animals. No rat was considered diabetic unless its fasting blood sugar concentration, determined by the Reinecke procedure (8), was greater than 250 mg. per cent.

Determination of P:O Ratios—Rat liver mitochondria were prepared by a modification (9) of the procedure of Schneider (6). All other manometric and chemical procedures were essentially the same as those reported previously (10). In the "long term" diabetic series, the following minor modifications were instituted: Potassium phosphate and substrate additions were increased to 50 and 30 μmoles, respectively, 10 per cent trichloroacetic acid was used for deproteinization, and an 8 minute period of thermal equilibration in the water baths was employed. In some cases in which the adenosine triphosphate (ATP)-creatine transphosphorylase system replaced glucose-hexokinase as a means of trapping the high energy phosphate (permitting higher concentrations of ATP to exist in the reaction mixture (9)), the following were added per vessel: 150 μmoles of creatine and 1 mg. of ATP-creatine transphosphorylase.

The addition of 30 μmoles of malonate to the reaction mixtures made possible the study of the following one- and two-step oxidations (10):

(1) α-Ketoglutarate → succinate

(2) Pyruvate → acetoacetate

(3) Caprylate → acetoacetate

(4) Glutamate → succinate

The inability of liver to metabolize significant amounts of acetoacetate made possible the study of the following one-step oxidation:

(5) β-Hydroxybutyrate → acetoacetate

The oxidation of succinate was examined in the absence of the Krebs cycle inhibitors.

Materials—The reagents used were as described previously (10). Glucagon-free insulin was generously donated by J. Lens, Organon Laboratories, Oss, Netherlands. Amorphous insulin was obtained from the Lilly Research Laboratories. ATP-creatine transphosphorylase was a highly

purified preparation from rabbit muscle made available by Dr. Kuby, Dr. Noda, and Dr. Lardy (11). Alloxan was purchased from the Eastman Kodak Company.

Results

"Short Term" Diabetic Mitochondria—Table I presents the results of experiments in which P:O ratios were compared, liver mitochondria being used from normal rats and from rats which had received alloxan injections 6 to 10 days prior to sacrifice. Most of the animals were studied 7 days after alloxan administration. While the average values are lower in the diabetic mitochondria with α-ketoglutarate and pyruvate as sub-

TABLE I

P:O Ratios of Normal and "Short Term" Diabetic Rat Liver Mitochondria

Substrate	No. of animal pairs	P:O,* normals	P:O,* diabetic	P:O of diabetic animals as per cent of P:O of paired control animals†
Pyruvate	10	2.6 (2.1–2.9)	2.0 (1.0–3.0)	104, 99, 95, 76, 76, 72, 71, 69, 51, 39
α-Ketoglutarate	8	3.8 (3.3–4.1)	3.1 (2.3–4.2)	102, 98, 93, 91, 86, 77, 68, 57
Succinate	4	1.5 (1.2–1.6)	1.6 (1.2–2.0)	125, 117, 107, 92

* P:O ratios are listed as average values, with the range of the P:O ratios indicated by the figures in parentheses.

† In each experiment a diabetic and a control rat were studied simultaneously. The figures indicate the P:O ratios of the individual diabetic animals with their paired control animals.

strates, no consistent alterations in the P:O ratios were observed among the individual animals.

With the method of administering alloxan used in these experiments, usually one-third to one-half of the animals succumbed between the 3rd and 10th day after treatment. The administration of 4 units per day of protamine zinc insulin did not improve the percentage of survival. This suggested that damage to tissues other than the pancreas is severe during this time interval and is consistent with earlier reports of the severe nephrotoxic and mild hepatotoxic action of this compound in rats (12, 13). A number of workers have reported the irregular occurrence of histological evidence of mild degenerative changes in liver parenchymal cells in the 1st week after alloxan treatment (12–15). This raised the question whether the occasional lowering of P:O ratios which we observed with "short term" diabetics may have been due to a direct toxic effect of alloxan

TABLE II

P:O Ratios of Normal and "Long Term" Diabetic Rat Liver Mitochondria

Substrate	Phosphate acceptor*	Preparation	No. of animals	P:O, average	Range
Pyruvate	Glucose	Normal	7	2.4	2.0 –2.8
"	"	Diabetic	7	2.1	1.7 –2.4
"	Creatine	Normal	4	2.0	1.8 –2.2
"	"	Diabetic	5	1.9	1.5 –2.2
α-Ketoglutarate	Glucose	Normal	10	3.1	2.75–3.6
"	"	Diabetic	7	3.3	3.1 –3.7
"	Creatine	Normal	1	2.4	
"	"	Diabetic	2	2.4	2.3 –2.5

*"Glucose" indicates the addition of glucose and hexokinase, and "creatine" the addition of creatine and ATP-creatine transphosphorylase, as described in the text.

TABLE III

*Addition of Insulin in Vitro to Rat Liver Mitochondria**

Experiment No.	Substrate	Phosphate acceptor†	Preparation	Insulin‡	P	O	P:O
				γ	μM	γ atoms	
1	α-Ketoglutarate	Glucose	Normal		17.4	5.5	3.2
	"	"	"	20	17.1	4.6	3.7
2	"	"	"		21.2	6.8	3.1
	"	"	"	20	19.0	5.6	3.4
3	"	"	Diabetic		19.2	5.9	3.3
	"	"	"	20	19.3	5.7	3.4
4	"	Creatine	"		16.0	6.5	2.5
	"	"	"	200	16.5	6.4	2.6
5	Pyruvate	"	"		12.3	5.6	2.2
	"	"	"	200	12.5	6.2	2.0

*"Long term" diabetic animals were used in Experiments 3, 4, and 5.

† "Glucose" indicates the addition of glucose and hexokinase, and "creatine" indicates the addition of creatine and ATP-creatine transphosphorylase, as described in the text.

‡ Glucagon-free insulin (Experiments 1, 2, and 3), amorphous insulin (Experiments 4 and 5).

on liver cells rather than to insulin deficiency.[2] Bailey, Bailey, and Hagan (15) have reported that, while mild fatty metamorphosis occurred in rab-

[2] In recent publications, M. U. Dianzani (16) has reported that mitochondria from fatty livers resulting from carbon tetrachloride or phosphorylated oil administration display markedly lowered P:O ratios and Dury (17) observed chemical alterations, including marked increases in the neutral fat content of livers from rats which had received alloxan injections from 4 to 72 hours prior to analysis.

bit liver in the first few days after alloxan treatment, no histological liver abnormalities could be detected after 2 to 5 months. Therefore, the examination of animals with long standing alloxan diabetes was indicated.

"Long Term" Diabetic Mitochondria—The effects of long standing diabetes (alloxan treatment from 3 weeks to 6 months prior to study) upon the P:O ratios of liver mitochondria are shown in Table II. No significant deviation from the ratios determined in normal rats was observed, either with the glucose-hexokinase or the creatine-ATP creatine transphosphorylase system used as the phosphate trap.

Addition of Insulin in Vitro—In view of the observations of Polis *et al.* (4) and Goranson and Erulkar (3) that insulin added *in vitro* increases the efficiency of oxidative phosphorylation, it was considered of interest to determine whether insulin could so affect preparations capable of high phosphorylative ability. As can be seen in Table III, the addition of insulin to normal or diabetic mitochondria caused no significant alteration of the P:O ratio. This is in agreement with the findings of Judah and Williams-Ashman (5) on preparations from normal animals.

Other Substrates—In addition to the substrates described above, the following were examined in a limited number of experiments: β-hydroxybutyrate, caprylate, and glutamate. No appreciable differences between normal and diabetic liver mitochondria were observed.

DISCUSSION

Liver was chosen as the tissue for study in these experiments because its metabolism is profoundly influenced by the diabetic state, and also because mitochondria may be isolated from this tissue in a readily reproducible manner and have been studied extensively in this laboratory (9, 10). The P:O ratios determined in these experiments are in satisfactory agreement with those reported earlier for normal animals (10).

While these studies have failed to detect any impairment arising from diabetes in the metabolic machinery by which mitochondria carry out tightly coupled oxidative phosphorylation, they have not ruled out possible effects on oxidative phosphorylation resulting from the interaction of mitochondria with other intracellular components. The examination of such interactions is beset with many uncertainties at both the technical and interpretive levels. For example, it has been observed by Ashmore *et al.* (18) and independently in our laboratory (19) that the glucose-6-phosphatase activity of alloxan-diabetic rat livers is markedly increased. In liver this enzyme is found exclusively in the microsomal fraction (20). The inclusion of liver microsomes in a P:O ratio determination reaction mixture would cause the release of inorganic phosphate from the hexokinase-produced glucose-6-phosphate resulting in an *apparent* P:O ratio which is lower than the true value. This effect would be much greater

with diabetic than with normal liver microsomes and therefore an even lower *apparent* P:O ratio would be obtained.

The authors wish to express their appreciation to Professor H. A. Lardy for advice and helpful criticism.

SUMMARY

1. P:O ratios for several substrates have been compared for normal and alloxan-diabetic rat liver mitochondria.

2. With "short term" diabetic rats (6 to 10 days after alloxan), no consistent alterations in P:O ratios were observed, although the average ratios were lower than those of the control rats. The liver mitochondria from an occasional animal displayed a marked lowering of the P:O ratio. This was possibly the result, not of insulin deficiency, but of the irregularly occurring mild hepatotoxic action of alloxan.

3. In long standing (3 weeks to 6 months) alloxan diabetes, no alteration in the efficiency of oxidative phosphorylation of liver mitochondria was detected.

4. Addition of insulin *in vitro* did not significantly affect the P:O ratios of normal or diabetic rat liver mitochondria.

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METABOLISM OF D-TRYPTOPHAN IN THE NORMAL HUMAN SUBJECT*

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About 10 years ago Albanese and Frankston reported that urines voided by normal human subjects who had been fed 1 and 2 gm. doses of DL-tryptophan showed very little greater output of tryptophan than did the urines of subjects fed L-tryptophan or unsupplemented normal diets (2, 3). They noted, however, that the former urines yielded a reddish purple precipitate when 0.1 N iodine solution was added to them (3), whereas urines excreted after the ingestion of L-tryptophan did not. They were unable to obtain the same precipitate with normal human urine to which they had added an unstated quantity of tryptophan (3), nor did any of the known tryptophan metabolites tested by them yield it. Hence they concluded that the reddish purple pigment must have been produced from an excreted "aberrant metabolite" which had been formed from the D component of the DL-tryptophan fed. From the nitrogen analysis and the partial absorption spectrum of the reddish purple precipitate they assumed that it was indigo red.

In their assays of urinary tryptophan, Albanese and Frankston used their own adaptation of the Jolles reaction for indican (2). This method indicates enormously greater daily outputs of tryptophan (281 ± 30 mg.) in normal urine (2) than do microbiological assays (4–8) or assays (9) based on the Shaw-McFarlane chemical procedure (10). In fact, the normal output indicated by this method approximates the 300 mg. of tryptophan found by Rose (11) to be entirely adequate for the maintenance of nitrogen balance in thirty-one adult subjects (male) and exceeds the 225 mg. per day found sufficient in fifteen by Baldwin and Berg (12).

The D enantiomorph of tryptophan is known to support nitrogen balance in the human subject only very poorly, if at all (11–13). Therefore, unless it is peculiarly susceptible to metabolic degradation, as Albanese and Frankston imply (3), it should be the more readily excreted isomer. There is some evidence in at least two reports that appreciably greater excretion

* The data in this paper are taken largely from a dissertation submitted in August, 1953, by Ralph R. Langner in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa. The work was supported in part by a grant from the Nutrition Foundation, Inc. A summary of the study was presented before the American Society of Biological Chemists at Atlantic City, April 13, 1954 (1).

of tryptophan does occur when DL- (12, 14) or D-tryptophan (12) is fed than when L-tryptophan is administered.

Chromatographic evidence outlined in this communication and assay by four chemical methods, including the Albanese-Frankston procedure, confirm previous findings that the administration of moderate single doses of DL- or D-tryptophan does promote the excretion of a relatively large portion in the urine of the normal human adult. The excretion of D-kynurenine and of traces of indolepyruvic acid and acetyltryptophan was also indicated. Formation of a reddish purple precipitate upon adding iodine to these urines was readily confirmed. Spectrophotometric evidence indicated that, contrary to the findings of Albanese and Frankston, the same pigment can be produced from normal urine to which tryptophan is added and even from properly buffered solutions of tryptophan in water. The reddish purple precipitate can also be obtained by the addition of bromine. Spectrophotometric comparisons show that the pigment is not indigo red.

EXPERIMENTAL

The D-tryptophan used in this study was prepared through resolution of the brucine salt of acetyl-DL-tryptophan[1] by the method of Shabica and Tishler (15). The $[\alpha]_D^{25}$, $c = 0.5$ gm. per 100 ml. of solution in water, was $+32.5$ to $+33.0°$ (16). The tryptophan was ingested at such a time as to allow use of the urine immediately after the 24 hour collection period. The urines were preserved with thymol and hydrochloric acid (2) and stored in the cold.

The urorosein employed in the spectrophotometric comparisons was prepared, as directed by Fearon and Boggust (17), from indolealdehyde made by the method of Boyd and Robson (18). Except where otherwise noted, the other compounds used in the chromatographic and spectrophotometric tests were from samples available from previous studies in the laboratory.

Chromatographic Analysis of Urine—Following the ingestion of 1 gm. of D-tryptophan, the urine responded to the bromine water test for free tryptophan and tasted sweet, as does D-tryptophan (19). Impure crystals of D-tryptophan were separated by precipitation of an aliquot of the urine with mercuric sulfate, followed by decomposition and isolation in the usual way. To obtain some indication as to what metabolites might also be present, an analogously collected urine specimen was concentrated to 10 to 20 ml. *in vacuo* (approximately 100-fold), and aliquots of 9.95 μl. were transferred to strips of Whatman No. 1 filter paper for chromatographing as directed by Mason and Berg (20). Ultraviolet light revealed a bright

[1] The acetyl-DL-tryptophan was kindly provided by The Dow Chemical Company of Midland, Michigan.

blue fluorescing spot (R_F 0.37) identical in position, color, and shape with the spot produced simultaneously by the slower moving D component of pure DL-kynurenine (21). A second very weakly fluorescing spot (R_F 0.48) and a faint tan fluorescing spot (R_F 0.65) were also observed. Comparative tests indicated that the last was apparently indolepyruvic acid. With ninhydrin the R_F 0.48 spot responded strongly. On control chromatograms tryptophan showed the same R_F value and the same color response. When several aliquots of urine were superimposed, the kynurenine spot obtained (R_F 0.37) also responded to ninhydrin, but overlapped the tryptophan spot. The ninhydrin color produced with the tryptophan darkened to a brownish purple, but that produced with kynurenine remained unchanged. Upon dipping analogous chromatograms into an alcoholic solution containing 0.5 per cent of *p*-dimethylaminobenzaldehyde and 5 per cent of hydrochloric acid and drying with heat, essentially as directed by Tabone *et al.* (22), the tryptophan spot gave a strong purple coloration, and the kynurenine spot was yellow. A faint purple spot was also observed at R_F 0.78. This coincides with the position noted for acetyltryptophan and suggests that some conjugation of tryptophan may have occurred. This spot did not respond to ninhydrin. The optical purity of the D-tryptophan ingested and the ready solubility of acetyltryptophan in the relatively large volume of 66 per cent alcohol used in recrystallizing the tryptophan seem to preclude the possibility that the acetyltryptophan could have had its origin in the diet. To judge from the chromatographic evidence, free tryptophan was the major excretory product.

Reaction with Iodine and Bromine—When iodine (0.1 N in potassium iodide solution) was added to the urines collected after the ingestion of D- or DL-tryptophan, they yielded the reddish purple precipitate noted by Albanese and Frankston (3). The pH values of these urines were around 5.6. The same purple-colored precipitate could be produced in normal urine having approximately this pH to which 30 mg. of DL-tryptophan had been added per 100 ml. Solutions of tryptophan in strongly acidic urines or in water gave a questionable response to iodine and usually yielded the brown-colored precipitates obtained by Albanese and Frankston and assumed by them to be diiodotryptophan (3). However, when the solution was first buffered and the iodine solution was then added slowly, the reddish purple precipitate predominated.

The conditions under which Albanese and Frankston (3) obtained their reddish purple precipitates seem to us to have been fortuitous. When the concentration of tryptophan is small, as in urines excreted after the ingestion of 1 and 2 gm. of DL-tryptophan, iodine can be added rapidly without forming the tan-brown precipitate produced by similar addition when the concentration of tryptophan is greater. Apparently two reactions

take place. In the one tryptophan is oxidized to yield the purple precipitate, and in the other the tryptophan is iodinated to yield the tan-brown precipitate. A violet color similar to that produced in the familiar bromine water test was invariably obtained when the iodine solution was added dropwise to a buffered solution of tryptophan (pH between 4 and 6), and relatively large amounts of tryptophan could be converted completely to the purple precipitate when these precautions were observed. After all of the tryptophan is oxidized, the brown iodine color persists. In the oxidative process 1 equivalent of iodine is consumed per mole of tryptophan, as judged by thiosulfate titration (23). When the purple precipitates were washed with water and dried *in vacuo* at 60° to constant weight (3), they were found to be free of iodine. The tan-brown precipitates contained iodine.

At the outset of these studies, bromine water was used routinely to determine qualitatively the presence of tryptophan in the urines excreted, and a tube set aside was observed to contain a precipitate strikingly similar to that obtained with iodine. Bromine was found to react over a wide pH range, but must also be added slowly to produce the reddish purple oxidation product instead of halogenated tryptophan. The absorption curves presented in Fig. 1 show the striking similarity of the purple precipitates obtained variously with iodine and with bromine. The absorption maxima are in the same region as that observed by Albanese and Frankston (3).

Some support of the evidence pertaining to the reaction of iodine with tryptophan to produce a purple precipitate is found in a short note by Fearon and Boggust (17), who call the product tryptophan purple. Of the several indole derivatives tested by them and by us, only tryptophan and tryptamine respond.

Comparison of Oxidation Product with Other Pigments—The purple pigment produced with iodine was assumed by Albanese (24) and Albanese and Frankston (3) to be indigo red. A sample of synthetic indigo red was therefore procured for comparison,[2] along with indigo blue, indigo carmine, and urorosein. The indigo red contained a small amount of indigo blue, which was first removed by the use of an alumina adsorption column. The absorption curve for indigo red, thus purified, showed a peak around 530 mμ in ethanol, and also secondary peaks around 285 and 245 mμ (see Fig. 2). The curves obtained for comparison on ethanol solutions of the reddish purple precipitates with iodine had a single peak at 540 mμ, with steadily and rapidly increasing absorption from 450 to 220 mμ. The curves for indigo carmine and indigo blue were even more divergent than that for indigo red. The absorption curve for urorosein was markedly different.

[2] We are indebted to Dr. A. T. Peters of the Department of Colour Chemistry of Leeds University in England for so graciously providing us this sample.

Rimington *et al.* (25) have reported the isolation of indigo red from the urine of sprue patients who had been fed high protein diets. When their method of isolation was applied to the urine excreted by a normal subject after the ingestion of D- or DL-tryptophan, no red pigment was obtained.

The synthetic indigo red provided us was insoluble in aqueous 10 per cent sodium hydroxide solution and soluble in chloroform. The dried

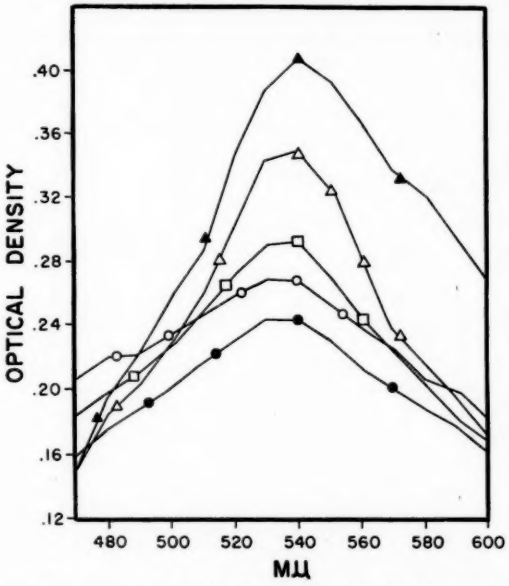

FIG. 1. Absorption curves of variously prepared reddish purple precipitates in acetone solution (10 mg. per 100 ml.). ▲, product obtained by the addition of iodine to normal urine to which 30 mg. of tryptophan had been added per 100 ml.; ●, precipitate obtained similarly by the addition of bromine; △, precipitation with iodine from urine excreted after the ingestion of 2 gm. of DL-tryptophan; ○, product prepared from similar urine with bromine; □, mixture of the four products listed above.

reddish purple precipitate was soluble in 10 per cent sodium hydroxide solution, but relatively insoluble in chloroform. The reddish purple precipitate decomposed fairly sharply on the Dennis melting point bar at 166°, the brown iodinated product (diiodotryptophan) decomposed at 204°, but the indigo red showed no decomposition at 300°.

The reddish purple precipitate had three bands, a red, a blue, and a violet, when dissolved in ethanol and passed through an alumina adsorption column. The three components could be separated by development with benzene, followed by a mixture of benzene and 95 per cent ethanol. Further characterization was not attempted.

Measurement of Tryptophan Output—The qualitative analyses described above leave little doubt that tryptophan itself is the chief indole derivative eliminated in the urine after the ingestion of DL- or D-tryptophan in the human subject and that it is the "aberrant metabolite" responsible for the production of the purple-red compound formed when iodine is added to the urine. Table I presents the quantitative estimation of added or excreted tryptophan by four chemical methods of analysis: (*a*) the Albanese-

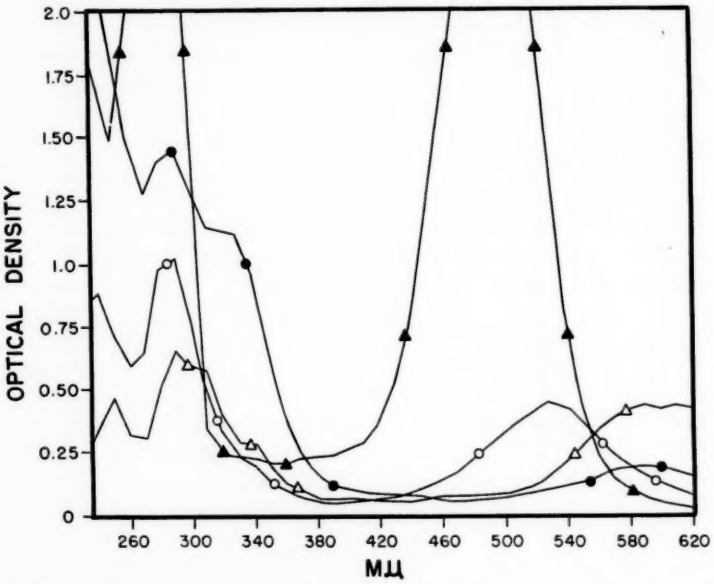

FIG. 2. Absorption curves of indigo red (○), indigo blue (●), indigo carmine (△), and urorosein (▲) in ethyl alcohol.

Frankston adaptation of the Jolles reaction for indican (2), (*b*) the Bates *p*-dimethylaminobenzaldehyde procedure (26), (*c*) the Eckert procedure of diazotization and coupling with *N*-(1-naphthyl)ethylenediamine (27), and (*d*) the Shaw-McFarlane adaptation (10) of the Hopkins-Cole glyoxylic acid reaction. The analyses were made on acid solutions of mercuric sulfate precipitates which had been prepared as directed by Albanese and Frankston from urines that had been extracted with ether and passed through a column of Permutit. The Klett-Summerson photoelectric colorimeter and the 540 mμ filter were used throughout. The tryptophan content was established by comparison of the readings with a standard curve. In all but the procedure of Albanese and Frankston, plots of

known tryptophan concentrations against readings gave approximately straight lines. The standard curve obtained with the Albanese and Frankston procedure was similar to the one published by them (2), and the 0.23 gm. per 24 hours found with their method in normal urine agrees well with their estimates of 226 to 336 mg. (2). The Bates, Eckert, and Shaw-McFarlane procedures are all more sensitive than is that of Albanese and Frankston.

TABLE I

Recovery of Added Tryptophan from Normal Urine and Apparent Excretion after Ingestion of D- *or* DL-*Tryptophan As Judged by Colorimetric Assay*

Tryptophan added to urine or ingested*	Albanese-Frankston		Bates		Eckert		Shaw-McFarlane	
	Found	Recovery	Found	Recovery	Found	Recovery	Found	Recovery
gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
0	0.23		0.05		0.03		0.02	
0.5	0.53	60	0.45	80	0.52	98	0.54	104
1.0	0.83	60	1.09	104	0.91	88	1.10	108
1.5	1.05	55	1.59	103	1.47	96	1.45	97
1.0 (D)	0.51	51			0.45	45	0.72	72
2.0 (D)	0.50	25	0.68	34	1.00	50	1.05	53
4.0 (DL)	0.59	15	0.83	21	1.06	27	1.32	33

* In the first column, 0 represents a control 24 hour urine sample excreted by a subject fed an average unsupplemented diet. The figures 0.5, 1.0, and 1.5 represent additions of tryptophan (per 2 liters of urine) made to aliquots of a previously analyzed control 24 hour sample which had been diluted to 2 liters. The recoveries recorded are net recoveries calculated on a 2 liter basis. The last three figures in the first column represent the ingestion of 1.0 and 2.0 gm. of D-tryptophan and of 4.0 gm. of DL-tryptophan. Recoveries in these instances are based on the analysis of 24 hour urine collections, but are not corrected for the average response which would have been obtained had no tryptophan been ingested.

Although there were wide variations on the same urine sample by the four methods, all have consistently indicated that an appreciable portion of the ingested D- or DL-tryptophan was excreted. Among different subjects, the percentage excreted, as judged by the Eckert and Shaw-McFarlane procedures, varied from 36 to 53 per cent after the ingestion of 2 gm. dosages of D-tryptophan.

Output of Kynurenine—Kynurenine was estimated by employing the same coupling agent as in the Eckert procedure, but only one-tenth as

much sodium nitrite was used and the diazotization was allowed to proceed for only 3 minutes instead of 30 (21). Application of this method to urines excreted after the ingestion of 2 gm. of D-tryptophan showed an apparent output of 80 to 200 mg. of kynurenine, presumed from the position of the spot on paper chromatograms (21) to be the D isomer. A similar output was estimated from the area of the spot on the chromatograms and the sensitivity of kynurenine to ninhydrin. A recent report by Dalgliesh and Tekman (28) indicates that both D- and L-kynurenine were detectable, the latter predominating, in urine excreted after the ingestion of 10 gm. of DL-tryptophan. In none of our tests with DL- or L-tryptophan has any L-kynurenine excretion been observed. Tryptophan showed no appreciable interference in the modification of the Eckert procedure outlined for the estimation of kynurenine. Kynurenine produces some interference in the Eckert procedure for tryptophan as routinely applied. Anthranilic acid interferes in both procedures (21), but was not detected on the paper chromatograms (20). The relatively small output of indolepyruvic acid and acetyltryptophan was not estimated quantitatively.

SUMMARY

Analyses by paper chromatography of urines voided by the normal human subject after the ingestion of D-tryptophan have shown that a considerable portion of the tryptophan is excreted, together with appreciable amounts of D-kynurenine and traces of indolepyruvic acid and acetyltryptophan.

Upon the addition of iodine, urines excreted after the ingestion of D- or DL-tryptophan yielded the reddish purple precipitates noted by Albanese and Frankston and assumed by them to have been produced from an "aberrant metabolite" of D-tryptophan. Tryptophan itself was found to respond to the iodine test, whether added to urine or dissolved in water. The conditions required to obtain the reddish purple precipitate are described. Comparative spectrophotometric tests indicate that the product can be obtained also by the addition of bromine, and that it is not indigo red.

Quantitative analyses by four different methods confirm the excretion of a large portion of the D-tryptophan ingested by the normal human subject as such or as a component of DL-tryptophan. The output in the urine is adequate to account alone for the reddish purple precipitate formed upon the addition of iodine.

Quantitative estimates of the kynurenine excretion were also made, but the output of indolepyruvic acid and acetyltryptophan was not assayed quantitatively.

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THE METABOLISM OF THYROXINE AND TRIIODOTHYRONINE IN THE EVISCERATED RAT

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The liver removes injected thyroxine very rapidly from the blood and excretes thyroxine or its derivatives in the bile. In 1919 Kendall (1) administered 200 mg. of thyroxine to a dog and found 43 per cent of the iodine in the bile within 50 hours. Albert and Keating (2), who administered 0.6 γ of radioactive L-thyroxine to young rats, found 30 to 43 per cent of the dose in the liver within 1 minute. This percentage then decreased at a rate proportional to the increase in radioactivity which occurred in the gastrointestinal tract. Only one-fiftieth as much I^{131} appears in the bile after the injection of radioactive inorganic iodide as after the injection of thyroxine (3). Gross and Leblond (4) have found that ligation of the common bile duct prior to injection of labeled thyroxine produced higher levels of radioactivity in liver and plasma with a lower level in the intestine. The normal physiologic excretory product in the bile was shown by Taurog, Briggs, and Chaikoff (5, 6) to be a conjugate, tentatively identified as the glucuronide. Studies of the formation of thyroxine glucuronide by perfused rat liver indicated that the liver was the chief site of synthesis of this compound, although kidney slices did show some ability to synthesize this compound (7). Roche, Michel, and Tata (8) have considered deiodination as another possible function of the liver in the metabolism of thyroxine, and recently Maclagan and Sprott (9) have shown that liver homogenates can deiodinate both thyroxine and triiodothyronine more rapidly than homogenates of extrahepatic tissues of the rat.

The liver also removes triiodothyronine rapidly from the blood (10–12), but Rawson and coworkers (13) have shown that this substance disappears from the liver much more rapidly than does thyroxine. The liver excretes triiodothyronine rapidly in the bile, both as the free amino acid and as the glucuroconjugate, along with a small amount of liberated iodide and an α-ketonic derivative, 3,5,3′-triiodothyropyruvic acid (8, 14).

To study further the rôle of the liver in the metabolism of thyroxine and triiodothyronine, we have investigated the changes which occur in the absence of the liver in the rat.

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XUM

EXPERIMENTAL

Adult male rats of the Sprague-Dawley strain, weighing from 165 to 420 gm. and maintained on Fromm's dog food containing 0.5 per cent iodized salt, were used in these studies. With the rats under ether anesthesia, the vena cava was completely ligated with surgical silk just above the kidneys so that adequate collateral circulation would be established. About 4 weeks later, with ether anesthesia, hepatectomy was done by evisceration, which included removal of the liver, stomach, intestines, spleen, and pancreas. Continuous collection of urine was provided by cystostomy with a small polyethylene catheter in these and in control rats, which were normal rats or rats with biliary fistula.

A single dose of from 20 to 30 μc. of radioactive[1] thyroxine, triiodothyronine, diiodotyrosine, or inorganic iodide was injected intravenously into each rat. The amount of thyroxine administered varied from 0.6 to 5.5 γ per rat except for two rats which received 12 γ each. The amount of triiodothyronine varied from 0.8 to 5.1 γ per rat. Diiodotyrosine was given in amounts varying from 80 to 188 γ; iodide was injected either carrier-free or with 1.6 to 3.3 γ of added carrier. A solution of a salt mixture and glucose was given by continuous intravenous injection throughout each experiment. Blood and tissues were removed at the end of the experiment. The tissues were frozen in dry ice, weighed, and crushed between chilled steel blocks. The total radioactivity of urine, plasma, and tissues was measured in a v ell-type NaI (Tl) scintillation counter and expressed as per cent dose per ml. of plasma and urine or per gm. of tissue. The radioactive iodine compounds were then extracted and separated by paper chromatography. The radioactive areas on the chromatograms were located by radioautographs on "no screen" x-ray film. The chromatograms were cut into areas, according to the location of the iodine compounds on the radioautographs. The radioactivity of each area was measured in the scintillation counter and the percentage of the radioactivity in each area was calculated.

Determination of the chloride content of urine was made by the method of Schales and Schales (15).

Extraction of Tissues—Plasma and other tissues, acidified with 0.2 ml. of 2 N HCl per ml. or gm., were extracted three times with 3 volumes of normal butanol (16). The crushed tissues were homogenized in the acidified butanol. Mean recoveries of the I^{131} present after the administration of radioactive thyroxine, triiodothyronine, and iodide to rats varied from 91.4 to 95.8 per cent, except for the 75.5 per cent recovery from the kidneys

[1] Obtained from the Abbott Laboratories with approval of the Atomic Energy Commission.

of rats which received NaI^{131} (Table I). An equal volume of chloroform was added to the butanol extract and the mixture was extracted three times with a volume of 2 N NH_4OH equal to one-third of that of the butanol extract. Mean recoveries in the ammoniacal extract of 85.3 to 93.8 per cent of the radioactivity of the butanol extracts were found, except for the plasma which contained large amounts of inorganic iodide, as in the rats which had received triiodothyronine 6 to 24 hours earlier, or NaI. An improved ammoniacal extraction of the I^{131} from rats which had received NaI was found if ethanol was substituted for butanol. The ammoniacal

TABLE I

Recovery of I^{131} from Tissues

No. of rats	Substance injected*	Hrs. after injection	Tissue	I^{131} recovery, per cent			TCA-soluble I^{131}, per cent
				Butanol extract	Ammonia extract	Total	
14	T4	3–20	Plasma	95.8 ± 0.6†	87.8 ± 2.5†	84.1 ± 2.2†	
7	"	8–20	Kidneys	94.3 ± 0.4	89.5 ± 2.4	84.5 ± 0.8	
10	T3	3	Plasma	93.8 ± 0.5	85.3 ± 2.1	79.9 ± 1.9	
18	"	6–24	"	93.6 ± 0.5	72.5 ± 3.1	67.9 ± 2.8	
16	"	3–21	Muscle	91.4 ± 0.8	88.2 ± 1.3	80.6 ± 1.2	
18	"	3–21	Kidneys	95.2 ± 0.8	90.4 ± 1.2	86.0 ± 1.3	
10	NaI	20	Plasma	93.9 ± 0.8	65.6 ± 2.9	61.5 ± 2.4	86.3 ± 1.4†
4	"	20	Kidneys	75.5 ± 1.5	91.2 ± 1.8	69.0 ± 2.1	66.2 ± 2.7
3	"	20	Lungs	91.6 ± 0.2	93.8 ± 0.3	85.9 ± 0.3	80.9 ± 3.1
4	"	20	Plasma	95.2 ± 0.2‡	96.8 ± 0.5	92.1 ± 0.6	

* T4 = thyroxine; T3 = triiodothyronine.

† Mean ± standard error.

‡ Ethanol extract instead of butanol.

extracts were evaporated to dryness *in vacuo*, dissolved in acid-butanol, and chromatographed. Awapara (17) and Salander and Patton (18) have shown that free amino acids of tissues can be extracted into water when an ethanol extract of tissue is shaken with chloroform and water. We have found that, if the water is made sufficiently alkaline, thyroxine and related compounds are also extracted from either ethanol or butanol and chloroform and thus removed from the tissue lipides which otherwise impair the resolution on chromatograms. We have used ammonia for the desired alkalinity, since it can subsequently be removed by evaporation *in vacuo*. Pigments and salts retained in the ammoniacal extracts may interfere with the resolution, particularly between inorganic iodide and the conjugate of thyroxine or triiodothyronine on chromatograms if extracts of tissue weighing more than 1 gm. are chromatographed.

Urine was chromatographed directly without preliminary extraction if 100 or 200 μl. contained at least 0.03 μc. of I^{131}. Otherwise, the urine was extracted with butanol or ethanol and the extract, at neutral pH, was dried *in vacuo*, dissolved in acid-butanol, and chromatographed.

Paper Chromatography—One-dimensional chromatograms on 10 by 12 inch sheets of Whatman No. 4 filter paper were developed by ascent in the solvent mixture butanol-dioxane (4:1) saturated with 2 N NH_4OH (19) in jars with 15 per cent sodium bisulfite present in a small beaker. The dioxane was redistilled to prevent decomposition of thyroxine.[2] Nonradioactive markers of inorganic iodide, thyroxine, and triiodothyronine were often streaked with the unknowns on the chromatograms. The inorganic iodide was located with palladous chloride (20) and the thyroxine and other iodinated tyrosine derivatives with the diazo reagent of Koessler and Hanke as used by Albright and coworkers (21). Some breakdown of thyroxine occurred during the development of the chromatograms, but the extent of this appeared to be fairly uniform. When the radioactive thyroxine, as obtained, was chromatographed in our system, from 85 to 90 per cent of the radioactivity was found in the thyroxine area, about 5 per cent in the inorganic iodide area, and about the same amount at the solvent front. A similar distribution was found when the thyroxine, added to tissue, was extracted and chromatographed. Thyroxine chromatographed and eluted from the thyroxine area also showed similar distribution when rechromatographed. Preparations of triiodothyronine, when chromatographed, showed 80 to 90 per cent of the radioactivity in the triiodothyronine area. Usually at least 2 to 5 per cent of the radioactivity was found in the iodide and thyroxine areas; in some preparations as much as 15 per cent of the radioactivity was found at the solvent front.

Results

Urinary Excretion of I^{131} by Rat—Normal rats excreted in the urine 18.2 per cent of I^{131} injected as radioactive thyroxine and 49.9 per cent of I^{131} administered as radioactive triiodothyronine within a period of 20 hours (Table II). In contrast, eviscerated rats excreted only 2.0 and 3.4 per cent of the I^{131} in the urine after the administration of these two substances in this time interval. Eviscerated rats excreted 22.0 per cent of I^{131} administered as sodium iodide and 29.7 per cent of I^{131} administered as diiodotyrosine, which is known to be rapidly deiodinated by the normal rat (22). The I^{131} output in the urine after the administration of iodide was only half as great as that in the control rats. This retention of iodide may be in part related to the much greater retention of chloride which occurred in these rats. Thus the hourly urinary output of chloride during the in-

[2] Owen, C. A., and McKenzie, B. F., unpublished data.

travenous injection of a saline solution averaged 0.0029 m.eq. with a standard error of 0.0005 in twenty-six samples of urine from twenty-two eviscerated rats, while the hourly output averaged 0.0702 m.eq. with a standard error of 0.0149 in twenty-one samples of urine from fourteen normal rats. The retention of I^{131} after the administration of radioactive thyroxine or triiodothyronine was much greater than that found after the

TABLE II

Radioactive Iodine after Injection of I^{131} Compounds

No. of rats	Substance injected*	Hrs. after injection	Urine		I^{131}, per cent of dose†	
			Volume	I^{131}, per cent of dose	Plasma	Muscle
			ml.			
4 N.‡	T4	3	4.7 ± 1.0§	3.8 ± 0.8§	8.2 ± 1.4§	11.4 ± 3.2§
7 "	"	20	21.1 ± 2.1	18.2 ± 1.8	6.5 ± 0.7	8.9 ± 0.9
4 E.	"	3	3.6 ± 1.4	0.5 ± 0.2	24.8 ± 0.4	25.7 ± 1.4
5 "	"	20	20.5 ± 2.4	2.0 ± 0.5	17.6 ± 1.6	24.5 ± 1.3
5 N.	T3	3	5.0 ± 0.7	4.0 ± 1.3	3.0 ± 0.7	21.4 ± 2.2
5 "	"	6	9.4 ± 2.4	15.6 ± 3.4	1.6 ± 0.2	15.1 ± 1.5
4 "	"	20	20.8 ± 2.5	49.9 ± 1.4	1.1 ± 0.3	6.9 ± 1.5
4 E.	"	3	3.7 ± 2.1	3.9 ± 2.1	3.6 ± 0.8	42.2 ± 3.4
5 "	"	6	7.4 ± 1.8	1.6 ± 0.5	4.2 ± 0.2	42.7 ± 5.4
9 "	"	20	24.1 ± 4.5	3.4 ± 1.0	7.0 ± 1.5	36.4 ± 2.9
1 N.	Ty2	21	12.4	70.7		
4 E.	"	20	31.8 ± 1.9	29.7 ± 5.6		
5 N.	NaI	20	16.7 ± 3.8	50.8 ± 7.3	1.6 ± 0.3	3.8 ± 0.8
11 E.	"	20	26.2 ± 3.6	22.0 ± 5.0	3.4 ± 0.4	11.6 ± 1.9

* T4 = thyroxine; T3 = triiodothyronine; Ty2 = diiodotyrosine.

† The plasma volume was assumed to be 3 per cent of the rat and the muscle mass 40 per cent.

‡ N. = normal rats; E. = eviscerated rats.

§ Mean ± standard error.

administration of either diiodotyrosine or iodide, indicative of decreased deiodination of the thyronines in the absence of the liver.

Radioautographs of urine from rats with livers, that is, normal rats or rats with biliary fistulas, showed that the I^{131} excreted after the administration of radioactive thyroxine was found predominantly as inorganic iodide. Radioactive thyroxine was found in normal urine in amounts usually less than 5 per cent of the total I^{131} excreted in the urine. The thyroxine conjugate found in normal urine accounted for less than 4 per cent of the I^{131}.

Radioautographs of twenty-one samples of urine from seven eviscerated

rats all showed the presence of free and conjugated thyroxine as well as inorganic iodide (Fig. 1). Free thyroxine accounted for from 17 to 40 per cent of the I^{131} and conjugated thyroxine from 4 to 13 per cent.

Following the administration of radioactive triiodothyronine, normal rats excreted I^{131} almost entirely as inorganic iodide. Only one radioactive band, that of iodide, was found on chromatograms in forty-one of

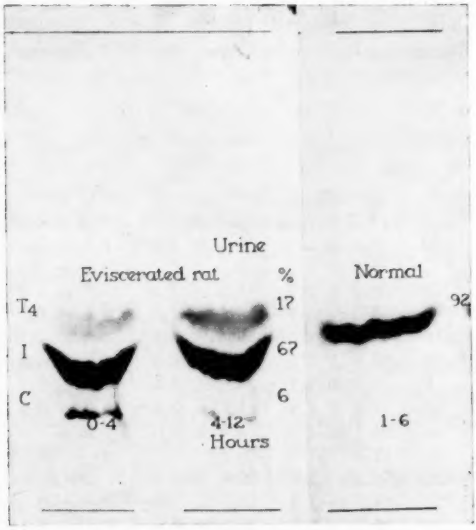

FIG. 1. Radioautographs of rat urine after administration of radioactive thyroxine. The sample was applied on the bottom line and the solvent front ascended to the top line. The areas of radioactivity found in the urine of the eviscerated rat were T4 (thyroxine), I (inorganic iodide), and C (thyroxine conjugate), while in the urine of the normal rat the only radioactive area was that of inorganic iodide. The eviscerated rat was injected intravenously with 28 μc. of thyroxine at 0 time. The normal animal similarly received 26 μc.

forty-five samples of urine from eight rats which were normal or had biliary fistulas, and this contained from 86 to 99 per cent of the I^{131}. Eviscerated rats excreted inorganic iodide which varied from 39 to 87 per cent of the I^{131} in the urine, but they also excreted free and conjugated triiodothyronine.

At least 75 per cent of the I^{131} excreted in the urine after the administration of radioactive diiodotyrosine to eviscerated rats was inorganic iodide.

Distribution of I^{131} in Tissues—Radioactive thyroxine, administered intravenously, disappeared from the plasma of the eviscerated rat more

slowly than from that of the normal rat. 3 hours after the administration of thyroxine, 50.5 per cent of the I^{131} was distributed in the plasma (24.8 per cent) and skeletal muscle (25.7 per cent) of the eviscerated rat, while a similar amount, 54.6 per cent, was distributed in the plasma, muscle,

TABLE III

I^{131} in Whole Organs

No. of rats	Hrs. after injection	I^{131}, per cent of dose						
		Brain	Heart	Kidneys	Lungs	Liver	G. I. tract*	Thyroid
Thyroxine								
4 N.	3	0.13	0.21	1.57	0.47	10.0	25.0	0.15
		±0.03	±0.04	±0.35	±0.10	±1.8	±4.4	±0.02
7 "	20	0.16	0.12	0.99	0.38	6.5	12.1	1.2
		±0.01	±0.01	±0.10	±0.06	±1.3	±2.4	±0.2
4 E.	3	0.34	0.41	4.75	1.16			0.12
		±0.03	±0.03	±0.55	±0.08			±0.01
3 "	20	0.22	0.33	2.80	1.1 (1)†			2.2 (1)
		±0.08	±0.02	±0.12				
Triiodothyronine								
5 N.	3	0.34	0.36	2.7	1.01	7.8	23.2	0.24 (1)
		±0.07	±0.03	±0.2	±0.19	±0.8	±3.0	
5 "	6	0.20	0.20	1.8	0.43 (2)	6.0	27.9	1.08 (2)
		±0.01	±0.02	±0.2		±0.6	±1.9	
4 "	20	0.08	0.08	0.6	0.23	2.7	13.8	3.28
		±0.01	±0.01	±0.07	±0.04	±0.8	±2.5	±0.29
4 E.	3	0.48	0.78	7.4	1.52			0.08
		±0.04	±0.03	±0.6	±0.19			±0.02
5 "	6	0.49	0.69	5.8	2.76			0.38
		±0.06	±0.11	±0.7	±0.56			±0.05
9 "	20	0.52	0.46	3.0	0.90			3.77
		±0.07	±0.10	±0.7	±0.09			±0.75

* Gastrointestinal tract with contents.

† The numbers in parentheses indicate the number of rats on which the corresponding determinations are based.

liver, and gastrointestinal tract of the normal rat (Table II). At least twice as much I^{131} was also found in brain, heart, and lungs, and 3 times as much in the kidneys of the eviscerated rat as in the normal rat (Table III). No difference was observed in the uptake of I^{131} by the thyroid. Higher levels of radioactivity were also found 20 hours after the injection of thyroxine in the tissues of eviscerated rats than in normal rats. The ratio of

the radioactivity of 1 gm. of tissue compared to that of 1 ml. of plasma, 3 hours after the injection of thyroxine, was similar in both normal and eviscerated rats, suggesting that the uptake of thyroxine by the other tissues was dependent on the level in the plasma (Table IV).

3 hours after the administration of radioactive triiodothyronine, 45.8 per cent of the I^{131} was found in the plasma (3.6 per cent) and skeletal muscle (42.2 per cent) of the eviscerated rat, while 55.4 per cent was distributed in the plasma, muscle, liver, and gastrointestinal tract of the normal rat. An increased amount of I^{131} was found in other tissues of the eviscerated rat as well as in skeletal muscle. There was a decrease in the

TABLE IV

Relative Distribution (Plasma = 100)*

No. of rats	Substance injected	Hrs. after injection	Brain	Muscle	Heart	Lungs	Kidneys	Liver
4 N.	T4	3	6 ± 1	10 ± 2	22 ± 1	34 ± 2	73 ± 8	101 ± 4
7 "	"	20	12 ± 1	12 ± 1	21 ± 1	39 ± 3 (5)†	74 ± 8	108 ± 14 (6)
4 E.	"	3	9 ± 1	8 ± 0.5	20 ± 1	45 ± 5	81 ± 8	
5 "	"	20	11 ± 0.5	16 ± 1	32 ± 2		89 ± 6	
5 N.	T3	3	55 ± 7	60 ± 8	140 ± 16	124 ± 12	483 ± 64	293 ± 25
4 "	"	20	32 ± 4	50 ± 5	68 ± 4	115 ± 11	256 ± 23	198 ± 14
4 E.	"	3	60 ± 2	86 ± 8	208 ± 5	227 ± 13	664 ± 61	
7 "	"	20	48 ± 7	50 ± 7	72 ± 6	105 ± 13	153 ± 15	

* Ratio of radioactivity of 1 gm. of tissue to 1 ml. of plasma multiplied by 100.

† The numbers in parentheses indicate the number of rats on which the corresponding determinations are based.

amount of I^{131} from the 3rd to the 20th hour in most of the tissues of the normal rat. A much smaller decrease in I^{131} was found in the skeletal and cardiac muscle, kidneys, and lungs of the eviscerated rat. The I^{131} content of the brain did not change; that of plasma increased. The ratio of I^{131} in 1 gm. of tissue compared to that in 1 ml. of plasma was much greater after the administration of triiodothyronine than thyroxine, largely because of the much lower amount present in the plasma. The tissues appear to bind triiodothyronine; hence it is not in equilibrium with the blood.

Fractionation of I^{131} in Tissues—Most of the I^{131} found in plasma and tissues 3 hours after administration of radioactive thyroxine to normal rats was present as thyroxine (Table V). Approximately two-thirds of the larger amount of I^{131} retained in skeletal muscle and kidneys of the eviscerated rat was also present as thyroxine. Radioactive inorganic iodide was present in all the tissues studied, indicating that iodine had been

liberated from some of the thyroxine in both normal and eviscerated rats. 20 hours after the administration of the labeled thyroxine the amount of I^{131} present in the tissues of the normal rat was too low for convenient fractionation, while in the eviscerated rat most of the I^{131} was still present as the substance injected. A radioactive substance which appears to be thyroxine conjugate because it has the same R_F as the thyroxine conjugate of bile, that is, an R_F lower than that of inorganic iodide but higher than

TABLE V

Partition of I^{131} 3 Hours after Administration of Thyroxine and Triiodothyronine

No. of rats	Tissue	I^{131}, per cent of dose		
		Total	Thyroxine	Iodide
Thyroxine				
4 N.	Plasma	8.2 ± 1.4	7.1 ± 1.2	0.4 ± 0.1
4 E.		24.8 ± 0.4	18.2 ± 1.9	1.9 ± 0.4
4 N.	Muscle	11.4 ± 3.2	7.2 ± 2.6	2.8 ± 0.6
4 E.		25.7 ± 1.4	16.7 ± 2.8	5.5 ± 1.3
4 N.	Kidneys	1.6 ± 0.4	1.2 ± 0.3	0.3 ± 0.1
4 E.		4.8 ± 0.6	3.2 ± 0.6	0.6 ± 0.2
Triiodothyronine				
			Triiodothyronine	
5 N.	Plasma	3.0 ± 0.7	1.5 ± 0.4	0.7 ± 0.2
4 E.		3.6 ± 0.8	1.5 ± 0.3	0.8 ± 0.2
4 N.	Muscle	21.4 ± 2.2	9.6 ± 0.7	4.8 ± 0.9
4 E.		42.2 ± 3.4	32.3 ± 0.4	2.7 ± 0.7
4 N.	Kidneys	2.7 ± 0.2	2.0 ± 0.3	0.4 ± 0.1
4 E.		7.4 ± 0.6	6.0 ± 0.5	0.5 ± 0.2

that of diiodotyrosine, was also found in the plasma of eleven of thirteen eviscerated rats and in four of twenty-one rats with livers. This substance was also present in the liver of the normal rat and was frequently observed in the skeletal muscle and kidneys of both normal and eviscerated rats and occasionally even in the heart and brain of these rats. The percentage of the radioactivity in this area of the chromatograms was usually less than 5 per cent in the normal rats and less than 10 per cent even in the eviscerated rats. There was often diffuse trailing of radioactivity in the triiodothyronine area, but in only a very few radioautographs of chromatograms of plasma and muscle did there appear to be a definite band of triiodothyronine.

About one-half of the small amount of I^{131} present in plasma 3 hours after administration of labeled triiodothyronine was triiodothyronine in both normal and eviscerated rats. Approximately three-fourths of the larger amount of I^{131} retained in skeletal muscle and kidneys of the eviscerated rat was also triiodothyronine both at 3 and at 20 hours after its administration. Deiodination of some of the triiodothyronine was shown by the presence of inorganic iodide in all the tissues of both normal and eviscerated rats. A radioactive substance with the same R_F as that of the triiodothyronine conjugate of bile was found in the plasma of eleven eviscerated rats and in eight of nineteen rats with livers. The conjugate was regularly found in radioautographs of extracts of skeletal muscle and kidneys of eviscerated rats, very frequently in normal rats, and occasionally also in cardiac muscle and brain of both eviscerated and normal rats. The percentage of the radioactivity in the conjugate area was usually less than 5 per cent in normal rats, but often as high as 10 to 15 per cent in the eviscerated rats. A small band of thyroxine was found in the radioautographs of plasma in ten of nineteen normal rats and in ten of eleven eviscerated rats. A band between the thyroxine and triiodothyronine markers which might be diiodothyronine was also found in the plasma of the eviscerated rats.

Comment

The 50 per cent reduction in urinary excretion of iodide, following administration of radioactive iodide, found in eviscerated rats compared to normal rats, as well as the 96 per cent reduction in urinary excretion of chloride which occurred simultaneously in the eviscerated rat, is undoubtedly part of the general metabolic response to surgery described by Moore and Ball (23) rather than a specific effect of removal of the liver. Moore and Ball found sodium retention associated with a normal excretion of chloride ion to be a common phenomenon in the convalescent patient. Wilkinson, Billing, Nagy, and Stewart (24) and others have found a retention of both chloride and sodium in patients during the postoperative period.

As much I^{131} was excreted as iodide in the urine by the eviscerated rat after the administration of radioactive diiodotyrosine as after the administration of iodide. This indicates that deiodination of diiodotyrosine was not appreciably altered by removal of the liver.

Very little I^{131} was excreted in the urine by the eviscerated rat after the administration of either radioactive thyroxine or triiodothyronine. Since the normal rat receiving these substances excreted I^{131} in the urine chiefly as iodide and since the eviscerated rat was able to excrete preformed iodide, these experiments suggested that the eviscerated rat was less able to form

iodide from the thyronines than the normal rat. Wilkinson and his associates (25, 26) attributed the decreased urinary excretion of I^{131}, which they found after the administration of radioactive thyroxine or triiodothyronine to rats when butyl 4-hydroxy-3,5-diiodobenzoate was injected simultaneously, to an inhibition of deiodination. Subsequently, Maclagan and Sprott (9) were able to show that homogenates of tissues did deiodinate the thyronines more slowly in the presence of this antithyroxine compound. Our observation that the I^{131} retained in plasma or tissues after the administration of the labeled thyronines to the eviscerated rat was present largely as the substance injected is further proof that deiodination of these substances is diminished in the eviscerated rat. The liver thus appears to be important in the formation of inorganic iodide from the thyronines, as well as in the excretion of these substances in the bile in the free and conjugated states. Such an interpretation of our data is complicated by the removal of other tissues with the liver in the evisceration. Studies in progress of the metabolism of radioactive thyroxine by the hepatectomized dog indicate, however, that deiodination of this subtance is also diminished when only the liver is removed.

Extrahepatic tissues of the eviscerated rat were able to deiodinate both thyroxine and triiodothyronine, as shown by the appearance of radioactive inorganic iodide in plasma and tissues. The rate of formation of the iodide was, however, slower than in the intact rat. These results are in keeping with the observation of Maclagan and Sprott (9) that homogenates of hepatic tissue deiodinate these thyronines more rapidly than homogenates of extrahepatic tissues. Albright, Larson, and Tust (27) have shown that kidney slices can deiodinate thyroxine to form triiodothyronine, Gross and Leblond (28) and Kalant, Sellers, and Lee (29) found a conversion *in vivo* of thyroxine to triiodothyronine in the mouse and rat, but Hogness, Van Arsdel, and Williams (30) were unable to demonstrate any interconversion *in vivo* of thyroxine and triiodothyronine in the rat. Kalant and coworkers (29) found triiodothyronine to be the principal radioactive component in muscle after the injection of thyroxine. In our experiments some interconversion of thyroxine and triiodothyronine was observed, as well as the probable formation of diiodothyronine from the triiodothyronine.

The more rapid disappearance of triiodothyronine than of thyroxine from the blood, which has been observed previously (10–12), was not altered by removal of the liver, while the normally slower disappearance of thyroxine was further decreased. The more rapid metabolism of triiodothyronine compared to that of thyroxine has been observed clinically by Rall and coworkers (10) and by Lerman (31), and in the rat by Wilkinson and Maclagan (32). Even in the eviscerated rat, in which the metabolism of both thyroxine and triiodothyronine was reduced, more I^{131} was excreted

by the rats receiving triiodothyronine, a higher percentage of this being inorganic iodide, than by those receiving thyroxine.

The presence of a conjugate of thyroxine in urine and tissues of the eviscerated rat which resembles the conjugate of thyroxine in bile (5, 6), in regard to both the R_F in butanol-dioxane-ammonia and hydrolyzability by bacterial β-glucuronidase, shows that conjugation of thyroxine can occur in extrahepatic tissues. It is of interest that, although this compound was also found in the normal rat, it was present in the plasma and extrahepatic tissues of the eviscerated rat in larger amounts. It is probable that this conjugate is the same as Compound 4 which Gross and Leblond (28) found in kidneys, liver, and possibly muscle.

SUMMARY

Deiodination of thyroxine was markedly reduced in the eviscerated rat, as shown by diminished excretion of iodide in the urine after administration of radioactive thyroxine and by prolonged retention of the thyroxine in plasma and tissues. Both free and conjugated thyroxine were excreted in the urine.

Deiodination of triiodothyronine was also markedly reduced in the eviscerated rat, although it disappeared as rapidly from the blood of these animals as from that of normal rats.

Deiodination of diiodotyrosine proceeded readily in the eviscerated rat.

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THE METABOLIC INHOMOGENEITY OF RIBONUCLEIC ACID PHOSPHORUS*

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The experiments to be described below were undertaken to investigate the extent to which the phosphate groups of liver ribonucleic acid (RNA) differ in turnover rate. Such intramolecular differences have been found in other types of macromolecules in the tissues. Steinberg and Anfinsen (1) reported varying turnover rates for a single amino acid located at several points in the molecule of ovalbumin, and Stetten and Stetten (2) found differences in the turnover rates of the glucose units situated in the central straight chain portion and the peripheral branches of glycogen. Bendich *et al.* (3) observed that the deoxyribose nucleic acid (DNA) of regenerating liver and of several normal tissues could be separated into two portions having different rates of formation; however, they were unable to separate the DNA of the liver of normal rats into two such portions.

The problem with respect to RNA is complicated by the often repeated observation (4–8) that the turnover rate of the phosphate groups of nuclear RNA is several times as great as that of the cytoplasmic RNA. The procedure used in the present experiments did not involve separation of the particulate fractions, but the differences in apparent turnover rate that were found are appreciably greater than any which have been reported between nuclear and cytoplasmic RNA. Also certain aspects of the data indicate that the observed changes in rate cannot be accounted for fully by the known higher turnover rate of nuclear RNA. The procedure used was to inject rats and rabbits with tracer phosphate, excise the livers 2 or 4 hours afterward, precipitate the proteins and nucleic acids with trichloroacetic acid (TCA), extract the acid-soluble P compounds, and then convert the RNA in the residue to water-soluble mixtures of mono- and oligonucleotides by prolonged treatment with successive portions of TCA solution at room temperature. The products formed were separated by ion exchange chromatography, and the relative specific activities of the P of these fractions were then determined.

EXPERIMENTAL

The experiments were carried out on male New Zealand white rabbits weighing from 1.8 to 2.4 kilos and on male white rats of the Wistar strain

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weighing from 300 to 350 gm. The tracer phosphate received from Oak Ridge was neutralized with $NaHCO_3$ and made up in 0.85 per cent solution of NaCl containing carrier Na_2HPO_4 equivalent to 5 γ of P per ml. The solution was injected intravenously into the rabbits and subcutaneously into the rats in dose of 50 to 200 μc. per kilo of body weight. 2 or 4 hours afterward, the livers were excised from the anesthetized animals, and immediately dropped into a large volume of a freezing mixture of dry ice and ether. Each rabbit liver was worked up separately; the livers of four rats were pooled. The frozen livers were broken up into small pieces in a tissue crusher chilled with dry ice and weighed, the proteins precipitated, and the acid-soluble P compounds extracted by treatment with 5 volumes of 10 per cent TCA solution in a Waring blendor in a cold room maintained at 1–2°. The residue was washed once with 3 volumes and once with 2 volumes of 5 per cent TCA solution to complete the removal of the acid-soluble P compounds.

The residue was brought to room temperature, and 5 volumes of 5 per cent TCA solution were added. The mixture was shaken at frequent intervals for 2 hours, centrifuged, and the residue washed with a small volume of the TCA solution. This treatment with 5 per cent TCA was repeated five times; the duration of the extraction period was 24 hours for the first four, and 72 hours for the final treatment. Each of the solutions obtained was freed of TCA by extraction with ether and made up to a suitable volume, and the optical density determined at 260 and 280 mμ in a Beckman model DU spectrophotometer. Although there was no reason to anticipate that DNA would undergo hydrolysis to soluble fragments in this treatment, tests were made with the diphenylamine reagent on all the extracts. These gave uniformly negative results. Less than 10 per cent of the material with absorption in the ultraviolet that was ultimately extracted was present in the first extraction (2 hours duration). The highest concentration of such material was found in the first 24 hour extraction, and progressively smaller amounts in the subsequent ones. Table I is a protocol of a representative experiment on a rabbit liver treated in this way.

When the extractions with 5 per cent TCA were begun immediately after the removal of the acid-soluble P compounds, the first two treatments yielded about one-third of the total extractable nucleotide material. However, when the RNA was extracted from the lipide-free residue of the TCA precipitate by the method of Davidson and Smellie (9), extraction with 10 per cent NaCl solution at 100°, or even when the TCA precipitate was dehydrated and freed of lipides before the treatment with TCA, over 80 per cent of the total nucleotide material was hydrolyzed to water-soluble fragments within 24 hours.

The solutions containing the soluble fragments were made ammoniacal, and the nucleotide material present was adsorbed on columns of Dowex 1 chloride 17 cm. high × 1.1 cm. in diameter. The resin used was the 2 per cent cross-linked material which Volkin and Cohn (10) found to give more satisfactory separation of the oligonucleotides than is possible with the ordinary resin with 10 per cent of cross-linking. About one-third of the material with absorption in the ultraviolet present in the first 2 hour extract and about 5 per cent of that in the first 24 hour extract failed to be adsorbed on the resin. It was found by paper chromatography in the butanol-ammonia-water system of Hotchkiss (11) that this contained adenine,

TABLE I

Degradation of Rabbit Liver RNA by 5 Per Cent Trichloroacetic Acid Solution

Each extract was made up to 500 ml. after removal of TCA, made ammoniacal, and the nucleotide material was adsorbed on Dowex 1 resin (chloride). Free bases and nucleosides were eluted with 500 ml. of 0.01 M NH_4Cl.

Extract No.	Duration of extraction	Optical density measurements					
		Extract		Run-off from resin column		0.01 M NH_4Cl eluate	
		260 mμ	280 mμ	260 mμ	280 mμ	260 mμ	280 mμ
	hrs.						
1	2	1.14	0.553	0.362	0.255	0.060	0.025
2	24	8.10	5.30	0.220	0.127	0.900	0.410
3	24	7.10	4.63	0.078	0.035	0.820	0.325
4	24	5.15	3.43	0.075	0.030	0.560	0.205
5	24	2.56	1.61	0.025	0.020	0.680	0.252
6	72	2.03	1.80	0.025	0.020	0.250	0.142

guanine, and a small amount of hypoxanthine. These two solutions also contained traces of orthophosphate, which was eluted from the columns by the mixture of 0.01 M $K_2B_4O_7$ and 0.025 M NH_4Cl used by Khym and Cohn (12) in their studies on the ion exchange separation of the phosphorylated sugars. The columns were then treated with 0.01 M NH_4Cl to remove bases and nucleotides (13). This treatment eluted about 10 per cent of the total material with absorption in the ultraviolet. Tests for ribose were negative; hence the material so eluted consisted of free bases. The elution of nucleotides was then begun by employing successively the eluents used by Cohn (14) for the mononucleotides and then the first six of the series used by Volkin and Cohn (10) for the separation of di- and oligonucleotides resulting from the action of ribonuclease on yeast RNA. This series of eluents removed over 90 per cent of the nucleotide material

on the columns, and the remainder was eluted with a small volume of 2 N HCl. The composition of the eluting solutions is given in Table II.

The progress of the solution was followed by measurement of optical density at 260 and 280 mμ in a Beckman model DU spectrophotometer. Each fraction representing a peak of optical density was concentrated by readsorption on a short column of Dowex 1 resin, followed by elution with a small volume of 1 N HCl. Measurement of optical density at 260 and 280 mμ was made, the solutions wet-ashed with nitric and sulfuric acids, the P precipitated with magnesia mixture, and the $MgNH_4PO_4$ precipitates converted to phosphomolybdate for counting. P determinations were made by the method of Fiske and Subbarow. The samples for counting contained from 250 to 350 γ of P. When necessary, carrier P was added to give this weight range. The phosphomolybdate precipitates were collected on specially prepared Pyrex Gooch crucibles and counted with a methane flow counter. Each sample was counted for a sufficient length of time to reduce the standard deviation of the count below 2 per cent. Duplicate counts were made when the time involved was not excessive.

Results

Only traces of mononucleotides were found in the first extract, and the total quantity of nucleotide material remaining on the column after elution of the free bases was too small for fractionation to be feasible. The entire quantity was therefore eluted with 2 N HCl. Mononucleotides in appreciable amount were found in all the other extracts. Well defined peaks corresponding to cytidylic acid were obtained from all these, and well defined peaks corresponding to adenylic acid in less than half. Peaks corresponding in elution characteristics and absorption spectra to uridylic and guanylic acids were obtained in only a few cases. However, considerable material was eluted with 0.002 N and 0.003 N HCl, the eluents for the mononucleotides, which did not correspond in absorption spectra to any of the common mononucleotides. In the first and second 24 hour extractions, this amounted to 5 to 15 per cent of the total material with ultraviolet absorption present in the extracts.

The elution of the di- and oligonucleotide materials did not give as clearly defined peaks as those obtained by Volkin and Cohn (10) in their separation of the products of the action of ribonuclease on yeast RNA, even though the ion exchange columns used by them were considerably shorter in relation to cross-section than those employed in the present experiments. The elution rate was about 50 ml. per hour, and fractions were collected for 2 hours. The ratio of optical densities at 280 and 260 mμ of successive fractions of the eluate representing the rising and falling phases of a peak were not necessarily constant. Nor did the ratios corre-

spond very well with those calculated from the molar extinction coefficients and the composition found by Volkin and Cohn for the corresponding peaks

TABLE II

Relative Specific Activities of P from Fragments of Liver RNA Separated by Ion Exchange Chromatography from Successive Treatments with 5 Per Cent Trichloroacetic Acid Solution

Liver of rabbit excised 4 hours after injection of P^{32}-phosphate. The values are in terms of counts per minute per mg. of P, calculated to the basis of 10^6 c.p.m. injected per kilo of body weight.

Eluent	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6
0.002 N HCl		662 (Cyt.)	396 (Cyt.)	88 (Cyt.)	Cyt.*	Cyt.*
		191 (Ad.)	†	61 (Ad.)	Ad.*	Ad.*
		†	33 (Urid.)	15 (Urid.)		
0.003 " "		†	33 (Guan.)	50 (Guan.)	87 (Guan.)	36 (Urid.)
			48	45		36 (Guan.)
0.005 " "		522 (Guan.)	†	†	†	
		1180		166		
0.01 " "			306	†	†	
			98			
			92			
0.01 " " + 0.0125 M NaCl			99			
0.01 N HCl + 0.025 M NaCl		481				
0.01 N HCl + 0.05 M NaCl		82	340			
0.01 N HCl + 0.1 M NaCl		654			33	147
		19				329
2 N HCl	890	46	92	173	44	41
0.002 N HCl	Adenosine-5'-phosphate from acid-soluble, 980					

Ad., adenylic acid; Cyt., cytidylic acid; Guan., guanylic acid; Urid., uridylic acid.
* P compound present with no absorption in the ultraviolet.
† Nucleotide material present, but no definite elution peak obtained.

in the elution pattern. For these reasons no attempt was made to establish the composition of the various peaks when these could not be calculated. Radioactivity determinations were made only on those fractions which gave well defined peaks of constant optical density ratio.

The first 24 hour extract yielded material with each of the series of eluents, and the second 24 hour extract with most of the eluents. In the subsequent extractions, however, only mononucleotides and those oligonucleotides requiring the highest concentrations of salt and acid for their elution were found.

The total P observed on wet ashing of the mononucleotide fractions from the last two extractions was many times that calculated from the optical density determinations. Evidently these fractions contained some of the substances without absorption in the ultraviolet which Davidson and Smellie (9) reported in their Schmidt-Thannhauser (15) digests. They subjected the livers of rabbits previously injected with tracer phosphate to this procedure, applied paper electrophoresis to the materials obtained, and made radioautographs of the paper strips. They found four spots on the radioautographs without corresponding absorption in the ultraviolet. Except for these fractions, the amounts of P in the mononucleotide fractions were within 2 per cent of those calculated from the optical density measurements. The amounts of P in the di- and oligonucleotide fractions corresponded within 10 per cent to a ratio of 1 atom of P per molecule of purine or pyrimidine base, assuming an average molar extinction coefficient of 10,675 at 260 mμ (10) for those fractions of which the composition was not determined.

The radioactivity data from a typical experiment are reported in Table II. These data were obtained from the liver of a rabbit sacrificed 4 hours after the injection of the tracer phosphate. The specific activity of the material in the first extraction (2 hours) was always among the highest in any of the RNA fragments. The outstanding finding in the other extracts was the random distribution of specific activities. While the highest specific activities were observed in the second extract, this same extract also yielded some fragments of very low specific activity. Also, some fractions of relatively high specific activity were obtained from that portion of the RNA least readily degraded into water-soluble fragments by treatment with TCA. The highest specific activities were equal to or higher than that of the P of the adenosine-5′-phosphate, which was isolated from the acid-soluble fraction of the P compounds by elution with 0.002 N HCl from a column of Dowex 1 resin.

DISCUSSION

Several investigators (4–8) have compared the specific activities of the P of nuclear and cytoplasmic RNA isolated from the livers of rats and rabbits 2 hours after the injection of tracer phosphate. The data are in general agreement that the specific activity and therefore the turnover rate of nuclear RNA-P under these conditions is some 10 to 25 times as

great as that of the P of cytoplasmic RNA. In all these studies, the RNA was isolated after conversion to mononucleotides by the Schmidt-Thannhauser (15) procedure or some modification thereof. The possibility of finding intramolecular differences in turnover rate was thereby limited to differences among the four mononucleotides, and these were rather slight. In the present experiments the range of specific activities was considerably greater than any which have been reported previously. Although some of the fragments of high specific activity must have been of nuclear origin and some of those of low specific activity must have been derived from the cytoplasm, it does not necessarily follow that these results can be accounted for entirely on such a basis, since the nuclei contain only about one-tenth of the total RNA of liver. In each of the experiments, at least one fraction was obtained of which the specific activity equaled or exceeded that of the adenosine-5′-phosphate of the acid-soluble fraction. In those studies in the literature in which the specific activities of RNA-P and acid-soluble P have been compared, the specific activity of RNA-P in the first few hours after injection of the tracer was only a small percentage of that of the acid-soluble P. For example, Smellie *et al.* (7) found that the maximal value for the specific activity of nuclear RNA-P was only about one-fifth that of the inorganic P of the liver, and that this maximum was reached 30 hours after the injection of the tracer. The specific activity of the acid-soluble P of rat liver reaches a maximum about 2 hours after the injection of the tracer (16) and falls slowly thereafter. The low values relative to the acid-soluble P found in Schmidt-Thannhauser digests therefore represent average values, which conceal the high degree of metabolic inhomogeneity shown by the RNA.

The present findings indicate that there is no necessary relation between the relative ease of enzymatic hydrolysis and resynthesis of specific phosphate linkages within the RNA molecule *in vivo* and the susceptibility of these linkages to the action of dilute acid. In this sense the degradation by acid represents a random process, in contrast to the action of ribonuclease. Markham and Smith (17) and Volkin and Cohn (10) found that the action of this enzyme on yeast RNA gave di- and oligonucleotides in which the terminal member, with a singly esterified phosphate group, contained a pyrimidine base. On the other hand, Merrifield and Woolley (18) reported that the terminal group in the dinucleotide products of the action of HCl on yeast RNA might be either a purine or a pyrimidine derivative.

These findings support the conclusion of Marshak and Calvet (4) that the phosphate groups of RNA are derived from some high energy phosphate compounds. The findings of RNA fragments with specific activities equal to or even exceeding that of the P of adenosine-5′-phosphate requires such an interpretation. The specific activity of this latter substance is

representative of those of the low energy P compounds of the acid-soluble fraction (16). This finding also indicates that the adenosine-5′-phosphate is not the direct precursor of the adenylic acid of RNA.

SUMMARY

1. The liver ribonucleic acid of rats and rabbits previously injected with tracer phosphate has been subjected to fractional degradation by treatment with dilute trichloroacetic acid solution at room temperature.

2. This treatment resulted in a gradual degradation of the RNA into water-soluble fragments, which were separated by ion exchange chromatography.

3. These fragments consisted of mononucleotides and larger molecules, presumably di- and oligonucleotides.

4. The ribonucleic acid degradation products isolated showed very wide variations in specific activity of their contained phosphorus.

5. The range of specific activities was in excess of those which have been reported by others between nuclear and cytoplasmic RNA-P.

6. The highest specific activities in these nucleotide fractions were equal to or greater than that of the P of adenosine-5′-phosphate isolated from the acid-soluble fractions of the same livers.

7. It is concluded that there is a high degree of metabolic inhomogeneity in the turnover of the phosphate groups of RNA and that the low turnover rates ascribed represent average values which conceal the presence of portions of the molecule with high turnover rates.

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THE QUANTITATIVE ESTIMATION OF AMINO NITROGEN BY DETERMINATION OF BOUND COPPER WITH THE FLAME PHOTOMETER*

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The measurement of combined copper has been used in a number of procedures for the quantitative determination of amino acids and peptides. Pope and Stevens (1) as well as Schroeder, Kay, and Mills (2) determined the combined copper iodometrically. Martin and Mittelmann (3) analyzed for copper with the polarograph. Woiwod (4) used the spectrophotometer to measure the color produced by the reaction of copper with diethyl dithiocarbamate. Spies and Chambers (5) described a simple spectrophotometric technique in which the blue color obtained when an excess of alanine is added to the combined copper is utilized. In this laboratory, the iodometric determination was found troublesome, the Woiwod procedure lengthy, and the Spies-Chambers method not reproducible when used on plant tissue that yielded colored or turbid hydrolysates.

The present paper describes a simple and rapid method for estimating amino nitrogen in protein hydrolysates in which the combined copper is measured with the flame photometer. The results obtained by the flame photometric method are compared with those obtained by a modification of the Woiwod method (4).

Reagents and Solutions

Copper chloride solution. 14 gm. of reagent grade cupric chloride dihydrate per 500 ml. of water solution.

Sodium phosphate solution. 68.5 gm. of reagent grade trisodium phosphate dodecahydrate per liter of water solution.

Borate buffer, pH 9.1 to 9.2. 57.3 gm. of sodium borate decahydrate per 3 liters of water; filtered.

Sodium diethyl dithiocarbamate. 1 gm. dissolved in 50 ml. of water.

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† From portions of a dissertation to be submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

The solution should be filtered; and refiltered before use if turbidity develops.

Washed copper phosphate. To 100 ml. of the sodium phosphate solution, 50 ml. of copper chloride solution were added with swirling, and the copper phosphate was removed by centrifuging. The residue was washed twice by resuspending in 150 ml. of sodium borate buffer and repeating the centrifugation. The copper phosphate was suspended finally in 250 ml. of sodium borate buffer, and 15 gm. of reagent grade sodium chloride were added to the suspension. This reagent was prepared according to the method of Schroeder *et al.* (2).

Copper standard solutions. A solution containing 2000 p.p.m. of copper was prepared by dissolving 2.682 gm. of reagent grade cupric chloride dihydrate in 500 ml. of distilled water. This solution was diluted to prepare standards containing from 1 to 40 p.p.m. of copper.

Isoamyl alcohol, Baker and Adamson, purified.

Amino acids were obtained from the following: DL-alanine and glycine, Eastman Kodak Company; L-arginine monohydrochloride, DL-aspartic acid, DL-histidine monohydrochloride, DL-isoleucine, DL-leucine, DL-methionine, and DL-threonine, Nutritional Biochemicals Corporation; L-glutamic acid, DL-phenylalanine, L-proline, DL-serine, DL-tryptophan, and DL-valine, General Biochemicals, Inc.; L-lysine, Merck and Company.

Phosphate buffer. 81.6 gm. of potassium dihydrogen phosphate and 23.0 gm. of sodium hydroxide were dissolved in 4 liters of water. The pH was adjusted to 8.4 with sodium hydroxide.

Protein Hydrolysates—Various plant tissues were hydrolyzed by a modification of the method of Melnick and Oser (6). A 1 gm. sample of the material to be hydrolyzed, 100 mg. of U. S. P. pancreatin (purified three times), and one or two crystals of thymol were placed in a 250 ml. beaker. 20 ml. of phosphate buffer and 40 ml. of water were then added to the beaker. The mixture was stirred 5 minutes as the pH was adjusted continuously to 8.4 with 5 per cent NaOH. The beaker was then covered with a watch-glass and set in a water bath maintained at 37°. The pH was readjusted to 8.4 at 12 hour intervals during hydrolysis. After the desired period of hydrolysis the hydrolysate was adjusted to pH 4.5 with glacial acetic acid and was boiled on a hot-plate for 3 minutes. The hydrolysate was then cooled, made to a volume of 100 ml., and filtered. After the filtrate was neutralized, the solution was ready for analysis.

Procedure

Flame Photometry—A Beckman model DU quartz spectrophotometer, equipped with a model 9200 flame photometry attachment and photomultiplier circuit, was used in the flame photometric work. The follow-

ing instrumental conditions were employed: wave-length 324.8 mμ, slit width 0.04 mm., oxygen pressure 16 pounds per sq. in., acetylene pressure 4 pounds per sq. in., maximal electrical sensitivity, sample feed rate, 1.56 ml. per minute.

The instrument was allowed to reach electrical stability before any analytical data were taken. The solutions to be analyzed were placed in 5 ml. beakers and atomized directly into the flame, and a series of three to five readings was taken on each sample. All transmittancy readings

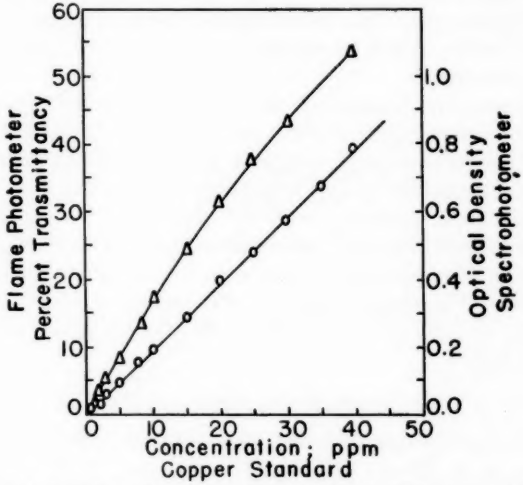

FIG. 1. Flame photometric (△) and spectrophotometric (○) copper standard curves

were corrected for a flame background with distilled water. Throughout this work, the background remained constant at 37 per cent transmittancy.

Flame Photometric Copper Standard Curve—This curve (Fig. 1) was prepared from data obtained by atomizing the copper standard solutions in the Beckman flame photometer. Per cent transmittancy, corrected for background, was plotted against copper concentration in parts per million to obtain the standard curve.

Spectrophotometric Copper Standard Curve—Portions of the same standards that were employed for flame photometry were used in preparing the spectrophotometric curve. The standards were diluted by pipetting 10 ml. of standard solution into 50 ml. volumetric flasks and making up to volume with water. To 10 ml. of this solution in a 50 ml. centrifuge tube equipped with a ground glass stopper was added 0.1 ml. of diethyl dithiocarbamate solution from a 1 ml. graduated pipette. 20 ml. of isoamyl

alcohol were added to the solution in the centrifuge tube, and the mixture was shaken vigorously for 45 seconds and then centrifuged 5 minutes at 2000 r.p.m. A portion of the alcohol layer was removed with a pipette and its optical density determined at 435 mμ. The calibration curve was obtained by plotting optical density, corrected for a water blank, *versus* the concentration of copper in the standard (Fig. 1).

Development of Complex—To 5 ml. of an amino acid solution or protein hydrolysate in a 15 ml. centrifuge tube were added 5 ml. of washed copper phosphate suspension. The tube was allowed to stand 5 minutes with occasional agitation and then centrifuged at 2000 r.p.m. for 5 minutes to remove the excess copper phosphate. A water blank also was carried through the reaction.

Flame Photometric Analysis—5 ml. of the supernatant liquid in the centrifuge tube were pipetted into a 50 ml. volumetric flask and made to volume with water. A portion of this solution was atomized into the flame photometer burner and its transmittance obtained.

Spectrophotometric Analysis—A 10 ml. aliquot of the solution used in the flame photometric analysis was diluted to a volume of 50 ml. 10 ml. of this solution were allowed to react with 0.1 ml. of diethyl dithiocarbamate solution. The reaction mixture was extracted with 20 ml. of isoamyl alcohol, and the optical density of the alcohol phase was measured as described above.

RESULTS AND DISCUSSION

Alanine Amino Nitrogen Standard Curve—Eleven alanine solutions containing from 10 to 400 mg. of amino nitrogen per liter were subjected to both the flame photometric and the spectrophotometric procedures. Alanine was chosen as the source of amino nitrogen, since Spies and Chambers (5) previously used it in expressing amino acid equivalence. The data obtained with these solutions were plotted *versus* the concentration of amino nitrogen to obtain the amino nitrogen standard curves shown in Fig. 2. The data also were referred to the proper copper standard curve (Fig. 1) to obtain the parts per million of copper bound, as shown in Table I.

From Table I it will be seen that alanine bound approximately theoretical amounts of copper over the entire range of amino nitrogen concentrations used. Theoretical binding is based on the observations of other workers (2–4, 7) that most amino acids, under conditions of this test, react to form compounds that can be represented by the formula CuA_2, where A equals an amino acid molecule.

Amino Acids—Sixteen solutions, each containing 300 mg. per liter of α-amino nitrogen of a given amino acid, were prepared. Also, a composite

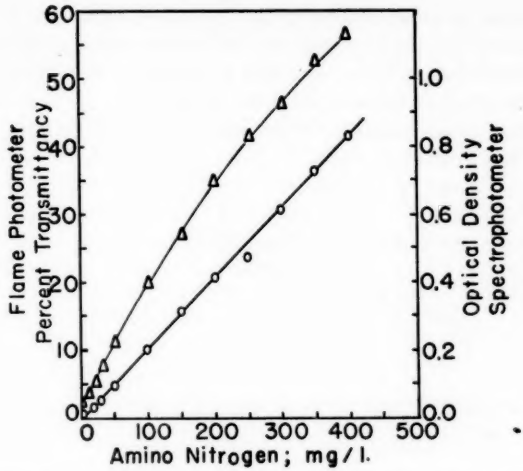

FIG. 2. Flame photometric (△) and spectrophotometric (○) α-amino nitrogen standard curves.

solution having the same amino nitrogen concentration was prepared in which the sixteen amino acids contributed equal amounts of α-amino nitrogen. The solutions were analyzed by both the flame photometric and spectrophotometric methods, and the readings obtained were referred to

TABLE I

Comparison of Copper Bound by Alanine Amino Nitrogen Determined by Flame Photometric and Spectrophotometric Methods

Amino nitrogen	Copper bound		
	Theoretical	Flame photometry	Spectrophotometry
mg. per l.	*p.p.m.*	*p.p.m.*	*p.p.m.*
10	1.1	1.5	1.0
20	2.3	2.3	2.0
30	3.4	3.5	2.8
50	5.7	5.5	4.9
100	11.4	11.3	10.5
150	17.0	16.0	16.0
200	22.7	22.0	21.3
250	28.4	27.8	26.5
300	34.1	32.0	31.7
350	39.7	38.3	37.8
400	45.4	42.3	43.0

the appropriate standard curve (Fig. 2). Good agreement between the two methods was obtained for all of the amino acids, as shown in Table II. The amount of α-amino nitrogen in histidine was abnormally high. Histidine bound approximately 90 per cent of the amount of copper required for the formula Cu_2A_3. This agrees with Schroeder *et al.* (2) and Spies and Chambers (5).

TABLE II

Recovery of α-Amino Nitrogen by Flame Photometric and Spectrophotometric Methods

Amino acid	Flame photometry	Spectrophotometry
	per cent	*per cent*
Alanine	100.0	100.0
Arginine monohydrochloride	100.7	95.7
Aspartic acid	97.7	102.3
Glutamic "	97.7	100.7
Glycine	100.7	100.7
Histidine monohydrochloride	128.0	126.7
Isoleucine	100.7	100.7
Leucine*	101.7	97.3
Lysine monohydrochloride	103.0	97.3
Methionine*	97.7	98.3
Phenylalanine*	97.7	101.3
Proline	97.7	94.3
Serine	100.7	102.3
Threonine	104.0	100.7
Tryptophan*	104.0	99.0
Valine	104.0	99.0
Composite	105.0	100.8

* The solution analyzed contained 2 parts of glycine nitrogen to 1 part of amino acid α-amino nitrogen.

Other workers (1, 2, 5) have found that phenylalanine, methionine, leucine, and tryptophan, when used alone, form insoluble copper complexes, but that they can be dissolved and separated from the excess copper phosphate by adding known amounts of glycine. In this work, 2 moles of glycine were added to each mole of the four amino acids listed above. No difficulty was encountered in the analysis of these amino acids under our conditions.

The results obtained with the composite solution of amino acids, without added glycine, indicate that insolubility of reaction products was not a problem in amino acid mixtures. This indicates the feasibility of determining mixed amino acids in protein hydrolysates by the flame photometric method. The recovery of 105 per cent α-amino nitrogen in the

composite mixture can be explained in part by the presence of histidine. The average recovery of amino nitrogen by the flame method as calculated from the data presented in Table II is 0.5 per cent above the theoretical without histidine or 2.2 per cent with histidine. This compares with an average recovery by the spectrophotometric process of 1.0 per cent above the theoretical with histidine and a −0.7 per cent without histidine.

Protein Hydrolysates—Eight protein-containing plant products were hydrolyzed enzymatically as described above. The hydrolysates were analyzed by the flame photometric and the spectrophotometric methods and the data obtained were referred to the proper amino nitrogen standard curve. Other workers (1, 5) have shown that when protein hydrolysates form complexes with copper those components which are least soluble

TABLE III

Comparison of Amino Nitrogen Found by Flame Photometric and Spectrophotometric Methods in Eight Protein Hydrolysates

Time hydrolyzed	Sample	Flame photometry	Spectrophotometry
hrs.		*mg. per l.*	*mg. per l.*
43	Dehydrated bromegrass	200	198
	Corn grain	60	53
	Cottonseed meal	318	300
	Hempseed meal	193	185
	Sorghum grain	105	98
	Soy bean meal	325	335
72	Dehydrated alfalfa	195	198
	Alfalfa silage	163	165

when by themselves are kept in solution by the others. Hence glycine was not added to any of the protein hydrolysates studied. Good agreement was obtained between the two methods (see Table III).

When hydrolysates of proteins contain a high concentration of the amino acids which tend to form insoluble copper complexes, it is theoretically possible that some amino acid is lost at the time of removal of excess phosphate. Even so, excellent agreement of results from these methods would still be obtained, just as in the case with the results reported in Table III, for the loss of amino acid would occur to an equal extent in the two procedures. However, others (1) have found that the Van Slyke method gives the same results that are obtained by a method which involves formation of copper complexes. It is assumed, therefore, on the basis of the good agreement reported in Table III that the results of the modification proposed here would be in good agreement with those by the Van Slyke procedure, at least with the hydrolysates tested.

Concerning Flame Photometric Method—Kingsley and Schaffert (8) have

shown that organic solvents, especially acetone, increase the sensitivity of the flame photometer to calcium, potassium, and sodium. In this work, acetone, dioxane, and ethyl alcohol were studied to determine their effect on the sensitivity of the flame photometer to copper. When the organic solvents were used in 50 per cent aqueous solutions, the corrected per cent transmittancy was increased 3-fold for acetone, 2½-fold for dioxane, and 2-fold for ethyl alcohol. The instrumental conditions employed with the organic solvents were the same as those when water alone was the solvent. However, organic solvents were not used in this work, since the amount of copper complex formed by the hydrolysates was sufficient to give adequate readings with the instrumental conditions and the dilutions employed.

The flame photometric method was found to be more rapid than the spectrophotometric method. The reaction with diethyl dithiocarbamate, the extraction with alcohol, and the accompanying centrifugation are eliminated. In analyzing for bound copper in the amino acids or protein hydrolysates, it is necessary to adjust instrumental conditions to reproduce the original calibration curve. In some instances it is known that organic material (9) or extraneous inorganic ions (10) may interfere in a flame photometric analysis. However, no difficulty was encountered in this procedure. This is substantiated by comparing the curve obtained by analyzing solutions containing only the copper ion in distilled water (Fig. 1) and that obtained by analyzing for copper bound with alanine (Fig. 2). When a blank mixture containing all the components except copper was analyzed under these conditions, a per cent transmittancy equal to that of distilled water resulted. All the usual precautions associated with good flame photometry techniques should be observed. During the course of analysis, it is occasionally necessary to clean the burner-atomizer assembly. In this study on amino nitrogen, it appears that the flame photometric method and the spectrophotometric method are equally reproducible.

SUMMARY

A simple, rapid method is described for the analysis of amino acids in which they form complexes with copper and the copper is measured with a flame photometer. Sixteen amino acids were studied. Results obtained by the flame photometric method and by a spectrophotometric method were compared. The application of the method to the determination of amino acids in enzymatically hydrolyzed plant tissue is described.

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ENZYMATIC DEALKYLATION OF AMINOPYRINE (PYRAMIDON) AND OTHER ALKYLAMINES*

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Many drugs which are alkylamines undergo dealkylation in the body. For example aminopyrine (2), ephedrine (3), codeine (4), meperidine (5, 6), methylamphetamine (7), and mephobarbital (8, 9) are all demethylated *in vivo*. The removal of ethyl groups has been demonstrated with quinacrine,[1] and an unusual type of dealkylation, the loss of a β-chloroethyl group, occurs in the biotransformation of *N*-dibenzyl-β-chloroethylamine (Dibenamine) (10).

In spite of the importance of dealkylation in drug metabolism little is known concerning the biochemical mechanism responsible for the removal of the alkyl groups: whether one or a number of enzyme systems are responsible for the dealkylation of various alkylamines; the relationship between structure of alkylamines and their resistance to dealkylation; and finally what function, if any, the dealkylating enzymes serve in normal metabolic processes.

Preliminary studies by Taggart, Poet, and Brodie[2] have shown that aminopyrine (Pyramidon, dimethyl-4-aminoantipyrine) is demethylated to 4-aminoantipyrine on incubation with slices or homogenates of rabbit liver. The present communication presents further studies on the enzymatic dealkylation of aminopyrine and related alkylamines. It will be shown that the dealkylation enzyme system is located in the microsomes of liver cells and requires both oxygen and reduced triphosphopyridine nucleotide. Evidence will be presented that the methyl groups removed from aminopyrine are converted to formaldehyde and that the ethyl group removed from the monoethyl analogue is converted to acetaldehyde.

Materials—Monomethyl-4-aminoantipyrine (henceforth referred to as MMAP) was kindly donated by Dr. Tainter of the Sterling-Winthrop Re-

* A preliminary report of this work was presented at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, April, 1953 (1).

[1] Taggart, J. V., unpublished data.

[2] Taggart, J. V., Poet, R., and Brodie, B. B., unpublished data.

search Institute. Diethyl-4-aminoantipyrine was synthesized by Mr. W. Lott of The Squibb Institute for Medical Research. The monoethyl, monobutyl, and dibutyl derivatives of 4-aminoantipyrine were prepared by Doris Titus. Diphenylpropylacetic acid (SKF acid) and its ester β-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525-A) were obtained through the courtesy of Dr. Glenn Ullyot of the Smith, Kline and French Laboratories. TPN (triphosphopyridine nucleotide), DPN (diphosphopyridine nucleotide), and DPNH (reduced DPN) were obtained in about 80 per cent purity from the Sigma Chemical Company. TPNH (reduced TPN) was prepared by the method of Kaplan *et al.* (11). Glucose-6-phosphate dehydrogenase was purchased from the Sigma Chemical Company. Glucose dehydrogenase was purified from beef liver by the method of Strecker and Korkes (12). Aminopyrine-C^{14} labeled in one of the two 4-*N*-methyl groups was synthesized by Doris Titus and had a specific activity of 1 μc. per μmole. The radioactive aminopyrine was diluted 1:40 with unlabeled compound.

Methods—Male albino rabbits[3] were stunned, exsanguinated, and the liver or other tissues immediately removed. Tissues were homogenized at 0–3° in 2 volumes of 0.2 M phosphate buffer, pH 7.4, with a Potter-Elvehjem type glass homogenizer. The preparations were usually used immediately, although no loss of activity resulted if they were stored in the cold for several hours.

In most studies of enzymatic dealkylation a 25 ml. Erlenmeyer flask contained 2.0 ml. of enzyme preparation, 100 μmoles of nicotinamide, 75 μmoles of $MgCl_2$, 0.2 μmole of TPN, 5 μmoles of alkylamine substrate, and water or 0.1 M phosphate buffer, pH 7.4, to give a final volume of 5.0 ml. The reactions were carried out in a metabolic shaking incubator at 37° for 1 hour in an atmosphere of air. At the end of the incubation period the reaction was stopped by the addition of 15 ml. of 6.7 per cent trichloroacetic acid to give a final concentration of 5 per cent trichloroacetic acid, and the precipitated protein was removed by centrifugation. Suitable aliquots, usually 1 to 5 ml. of the supernatant solution, were assayed for remaining substrate or for metabolic product.

The disappearance of MMAP or aminopyrine could not be used as a measure of dealkylation, since appreciable amounts of these substrates disappeared by an alternative pathway (see below). Dealkylation activity was therefore determined by measuring the amount of 4-aminoantipyrine formed by the dealkylation of aminopyrine or its homologues. Under the

[3] Rabbit liver homogenates had considerably higher dealkylation activity than liver homogenates of rat and guinea pig with aminopyrine or monomethyl-4-aminoantipyrine as substrates. For this reason the studies described in this paper were made with rabbit liver.

experimental conditions described, 4-aminoantipyrine is metabolized to a small extent, less than 10 per cent, by whole rabbit liver homogenate, but is stable when incubated with microsomal preparations of liver. In most experiments MMAP was used as the substrate, since the demethylation of this compound proceeded about 4 times as rapidly as aminopyrine, its dimethyl analogue.

Analytical Methods—4-Aminoantipyrine was assayed directly in a trichloroacetic acid filtrate of the various enzyme preparations by diazotization and coupling with α-naphthol, as described by Brodie and Axelrod (2). As applied to liver homogenates incubated with aminopyrine or MMAP, it was presumed that only 4-aminoantipyrine and not any other metabolite was measured, according to the following criteria: The compound determined had a free amino group, since it could be diazotized and coupled with α-naphthol; the value obtained by direct assay of the filtrate was the same as that obtained by the method involving preliminary extraction of the compound with chloroform (2); and finally the azo derivative formed in the analytical procedure when subjected to ascending chromatography on Whatman No. 1 paper, with butanol saturated with 0.5 N HCl as the solvent system, yielded a single spot having the same R_F value, 0.78, as the authentic derivative.

Aminopyrine was determined spectrophotometrically after its extraction into ethylene dichloride (2). MMAP was estimated in the same manner as that described for aminopyrine.

Formaldehyde was determined by the chromotropic acid method of MacFadyen (13) as modified by Mueller and Miller (14), except that the formaldehyde was distilled from the deproteinized incubation mixture acidified to a final concentration of 20 per cent trichloroacetic acid instead of with perchloric acid.[4] Formaldehyde was trapped by including 0.01 M semicarbazide in the incubation mixture. This concentration of semicarbazide did not inhibit enzymatic dealkylation. Zero time control flasks containing liver homogenate with added aminopyrine or MMAP contained no measurable formaldehyde, and incubated control flasks without substrate yielded less than 0.1 μmole of formaldehyde.

Acetaldehyde was trapped with 0.01 M semicarbazide and determined after distillation from a tungstic acid filtrate according to the method of Stotz (15).

Estimation of $C^{14}O_2$ and Formaldehyde-C^{14} in Enzyme Preparations—The incubation of aminopyrine-C^{14} with liver homogenates was carried out in closed vessels, and the carbon dioxide evolved was trapped as carbonate in 20 per cent KOH contained in a center well. At the end of the incubation period trichloroacetic acid was added to the incubation mixture to liberate

[4] Axelrod, J., personal communication.

bound carbon dioxide. The carbonate in the well was precipitated as barium carbonate, and the radioactivity was determined with a low background gas flow counter, corrected for self-absorption.

To determine formaldehyde-C^{14}, 5 mg. of non-radioactive formaldehyde were added to the deproteinized incubation mixture which was made free of radioactive carbon dioxide by flushing with CO_2. The pH was adjusted to 4.7, and 20 ml. of 0.4 per cent of dimedon (1,1-dimethyl-3,5-diketocyclohexane) were added. After 24 hours, the dimedon formaldehyde derivative was filtered, recrystallized from ethanol, and the derivative plated and counted. Recrystallization from ethanol was repeated until constant specific activity was obtained. Radioactivity was calculated as counts per minute per micromole after correction for self-absorption.

EXPERIMENTAL

Distribution of Dealkylation Enzyme System in Various Tissues—1.0 μmole of aminopyrine was incubated with about 500 mg. of slices of rabbit liver, skeletal muscle, kidney, intestinal mucosa, and spleen in 3.0 ml. of Krebs-Ringer-phosphate buffer at pH 7.4. Only liver was found to demethylate aminopyrine; about 0.1 μmole of 4-aminoantipyrine was formed by this tissue in 1 hour.

Requirements of Enzyme System in Liver Homogenates—Preliminary experiments showed that unless nicotinamide was added very little demethylation of MMAP to 4-aminoantipyrine occurred in homogenates of rabbit liver. This suggested that DPN or TPN might be required, though the addition of these coenzymes did not increase the extent of demethylation in the presence of nicotinamide. A definite requirement for TPN was demonstrated after decreasing the endogenous supply of pyridine nucleotides by preincubation of the homogenate without added nicotinamide at 37° for 8 minutes. The activity of the homogenate was reduced by about half by this procedure, but could be restored by the addition of TPN but not by DPN.

Further evidence that TPN was essential for dealkylation activity was obtained after dialysis of the homogenate overnight at 6° against 0.02 M phosphate buffer, pH 7.4. The resulting loss of activity could be partially restored by the addition of TPN but not by DPN. Other requirements were also evident after dialysis of the homogenate (Table I). Mg^{++} increased the activity of the dealkylation system, though the concentration required for maximal activity was rather large, about 0.015 M. The addition of glucose-6-phosphate also stimulated the activity, nearly doubling the extent of dealkylation. Mg^{++} and glucose-6-phosphate also increased the activity of the undialyzed homogenate, but to a much smaller extent.

Oxygen was found to be required for dealkylation activity. For example

when 5 μmoles of MMAP and the supplemented liver homogenate were incubated with air or oxygen as the gas phase, 1.76 and 1.75 μmoles, respectively, of 4-aminoantipyrine were formed. In contrast, 0.06 μmole was produced in the reaction carried out in an atmosphere of nitrogen.

Variation of the pH of the incubation medium showed that optimal activity occurred in the range of 6.8 to 7.5 and was considerably reduced above or below these values.

The amount of 4-aminoantipyrine determined after various periods of incubation showed that no further demethylation took place after 1 hour with amounts of substrate ranging from 1 to 5 μmoles. At this time about one-third of the substrate had been dealkylated. On the addition of more

TABLE I

Requirements for Enzymatic Demethylation of Monomethyl-4-aminoantipyrine by Dialyzed Liver Homogenate

2 ml. of liver homogenate were dialyzed overnight against 0.02 M phosphate buffer and then incubated for 1 hour at 37° with 5 μmoles of MMAP and additions, as indicated, in a final volume of 5 ml.

Nicotinamide, 100 μmoles	TPN, 0.2 μmole	Mg^{++}, 75 μmoles	Glucose-6-phosphate, 15 μmoles	DPN, 0.2 μmole	4-Aminoantipyrine formed
					μmoles
−	−	−	−	−	0.06
+	+	−	−	−	0.64
+	+	+	−	−	0.87
+	+	+	+	−	1.54
+	−	+	+	+	0.26

substrate no further activity was observed, indicating that the enzyme system had been inactivated during the incubation period.

Location of Dealkylation Enzyme System within Liver Cell—Rabbit liver homogenates were centrifuged at 9000 × *g* for 10 minutes to sediment nuclei and mitochondria. Dealkylation activity was found to remain entirely in the supernatant fraction which contained the microsomes and the soluble enzymes (Table II).

The intracellular location of the dealkylation enzyme system was determined more precisely by fractionating rabbit liver homogenates by differential centrifugation with isotonic sucrose, as described by Schneider and Hogeboom (16). The microsomal fraction was washed once with isotonic sucrose and suspended in 0.1 M phosphate buffer, pH 7.4, and the soluble fraction was dialyzed overnight at 6° against 0.01 M phosphate buffer, pH 7.4. Assay of the cellular fractions for dealkylation activity indicated that components of both the microsomal and soluble fractions were necessary for dealkylation activity (Table II).

Rôle of Soluble Fraction—The requirement for TPN and oxygen in the dealkylation system at first suggested that removal of the methyl group involved dehydrogenation, TPN acting as a hydrogen acceptor. But, as shown in Table I, dialyzed liver homogenate produced much lower dealkylation activity if glucose-6-phosphate was omitted. In addition, the soluble fraction of the cell contained considerable glucose-6-phosphate dehydrogenase activity as measured spectrophotometrically (17). This suggested that TPN was converted in the complete system to the reduced form. Experiments were therefore undertaken to see whether the actual cofactor was TPNH. Washed microsomes were incubated with MMAP,

TABLE II

Cellular Localization of Enzyme Activity

Cell fractions were incubated with 5 μmoles of monomethyl-4-aminoantipyrine as described under "Methods," except that 30 μmoles of glucose-6-phosphate were also added.

	4-Aminoantipyrine formed
	μmoles
Whole homogenate*	1.40
Supernatant fraction†	1.45
Microsomes‡	0.05
Soluble fraction of supernatant§	0.05
Microsomes + soluble fraction	1.45

* Homogenate (equivalent to 0.66 gm. of liver) prepared in phosphate buffer, pH 7.4, as described in "Methods."

† Supernatant fraction (equivalent to 0.66 gm. of liver) prepared by centrifugation of homogenate for 10 minutes at 9000 × *g* to remove nuclei and mitochondria.

‡ Microsomes (equivalent to 0.66 gm. of liver) prepared in isotonic sucrose (16).

§ 2 ml. of dialyzed soluble fraction equivalent to 200 mg. of liver.

glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and TPN in the absence of the soluble fraction of the cell. Under these conditions high dealkylation activity was observed (Table III). The soluble fraction could also be replaced by glucose dehydrogenase, glucose, and TPN. Direct evidence that TPNH was involved in dealkylation was obtained when the addition of chemically prepared TPNH to MMAP and washed microsomes effected the formation of 4-aminoantipyrine (Table III). As would be expected, DPNH could not replace TPNH in these experiments. It may be concluded, therefore, that the dealkylation enzyme system is located in the microsomal fraction of the liver cell and that the soluble fraction participates in the over-all reaction by maintaining TPN in its reduced form.

The effect of Mg^{++} ion was measured in a system consisting of microsomes, MMAP, and TPNH. Equivalent dealkylation activity was ob-

served with or without the addition of 75 μmoles of Mg^{++}. It may be presumed, therefore, that the stimulation previously noted in liver homogenates by Mg^{++} is due to an effect of the ion on the enzyme system which produces TPNH in the soluble portion of the cell.

Other Cofactors Tested—The following cofactors failed to increase activity when added to the liver homogenate system containing monomethyl-4-aminoantipyrine: 30 γ of flavin adenine dinucleotide, 200 γ of flavin monophosphate, 15 γ of folic acid, 5 μmoles of ascorbic acid, 4 γ of vitamin

TABLE III

Requirement for TPNH in Dealkylation of Monomethyl-4-aminoantipyrine by Liver Microsomes

Microsomal preparations equivalent to 1 gm. of liver were incubated as described under "Methods," except that 30 μmoles of glucose-6-phosphate were also added.

Components	4-Aminoantipyrine formed
	μmoles
Microsomes + 2 ml. dialyzed soluble fraction + 0.2 μmole TPN	2.21
" + 0.2 μmole TPN	0.12
" + 0.2 " " + glucose-6-phosphate dehydrogenase	1.46
Microsomes + TPNH*	0.90
" + DPNH*	0.0

* A total of 7 μmoles of the reduced pyridine nucleotides was added to the incubation system, 2 μmoles at zero time and 1 μmole at 10 minute intervals thereafter, since both reduced coenzymes disappeared rapidly even in the absence of substrate. Mg^{++} and glucose-6-phosphate were omitted.

B_{12}, 15 units of coenzyme A, 50 μmoles of cysteine, and 5 μmoles of glutathione.

Inhibitors of Dealkylation—The dealkylation system was not inhibited by cyanide, azide, fluoride, or iodoacetate at 1×10^{-3} M concentration. Cytochrome *c*, 4×10^{-5} M, and vitamin K, 1×10^{-3} M, produced about 30 per cent inhibition. 10 μmoles of pyruvate, α-ketoglutarate, or citrate had no inhibitory effect. However, diphenylpropylacetic acid and its ester diethylaminoethyl diphenylpropylacetate (SKF 525-A), which have been shown to inhibit the biotransformation of a large variety of drugs (18, 19), exerted a marked inhibition. At 2×10^{-4} M concentration these compounds inhibited the demethylation of aminopyrine and MMAP by about 50 per cent.

Substrate Specificity—The metabolic removal of alkyl groups by rabbit liver homogenate was compared in a series of aminopyrine homologues.

The data in Table IV show that the monosubstituted compounds are more easily dealkylated than the disubstituted homologues and that the extent of dealkylation decreases with increasing size of the alkyl group.

Products of Reaction—The fate of the methyl groups removed from aminopyrine was determined by radioactive and chemical procedures. In preliminary experiments 5 μmoles of C^{14}-methyl-labeled aminopyrine containing 57,000 c.p.m. were incubated with the supplemented rabbit liver homogenate preparation to determine whether the methyl groups were oxidized to carbon dioxide. $C^{14}O_2$ equivalent to about 75 per cent of the methyl groups removed from aminopyrine (as calculated from the amount of 4-aminoantipyrine formed) was recovered. The deproteinized incubation mixture was analyzed for labeled formaldehyde and was found to

TABLE IV

Dealkylation of Various Analogues of Aminopyrine by Liver Homogenate

5 μmoles of each alkylamine were incubated with liver homogenate as described under "Methods."

Substrate	4-Aminoantipyrine formed	Per cent of substrate dealkylated
	μmoles	
Monomethyl-4-aminoantipyrine	1.93	36
Dimethyl-4-aminoantipyrine (aminopyrine)	0.62	11
Monoethyl-4-aminoantipyrine	0.60	11
Diethyl-4-aminoantipyrine	0.29	6
Monobutyl-4-aminoantipyrine	0.56	9
Dibutyl-4-aminoantipyrine	0.10	1

contain about 5 per cent of the methyl group activity as this compound. The presence of traces of formaldehyde raised the possiblity that the methyl groups in aminopyrine were converted to formaldehyde, the major portion of which then underwent oxidation to carbon dioxide.

Conclusive evidence that formaldehyde was a primary product in the demethylation of MMAP in the rabbit liver homogenate was obtained by trapping formaldehyde with 0.01 M semicarbazide. At the end of the incubation period the trapped formaldehyde was found to be equivalent to the 4-aminoantipyrine formed (Table V). 2 moles of formaldehyde were found for each mole of 4-aminoantipyrine produced with aminopyrine as substrate, indicating that both methyl groups of this compound are converted to formaldehyde.

The results in Table V show that about twice as much MMAP or aminopyrine disappeared as could be accounted for by the appearance of 4-aminoantipyrine or formaldehyde, indicating that another metabolic pathway

for these substrates is present in liver. Preliminary work reveals that the system which catalyzes this unknown pathway is also present in microsomes.

By analogy from the fate of the methyl groups, it might be expected that an ethyl group attached to nitrogen would be converted to acetaldehyde. The fate of the ethyl group in monoethyl-4-aminoantipyrine was studied after incubation of 5 μmoles of substrate in liver homogenate in the presence of 0.01 M semicarbazide. The aldehyde arising from dealkylation of monoethyl-4-aminoantipyrine was identified as acetaldehyde by determination of the absorption spectrum of its derivative with *p*-hydroxydiphenyl (15). Authentic samples of formaldehyde, acetaldehyde, and glycolaldehyde after reaction with this reagent gave characteristic absorption peaks at 605, 560, and 585 mμ, respectively. Glyoxylic acid did not

TABLE V

Formation of 4-Aminoantipyrine and Formaldehyde from Monomethyl-4-aminoantipyrine and Aminopyrine by Liver Homogenate

5 μmoles of substrate were incubated with liver homogenate as described under "Methods."

Substrate	4-Aminoantipyrine formed	Formaldehyde formed	Substrate disappeared
	μmoles	*μmoles*	*μmoles*
MMAP	1.1	1.2	2.2
Aminopyrine	0.35	0.70	0.75

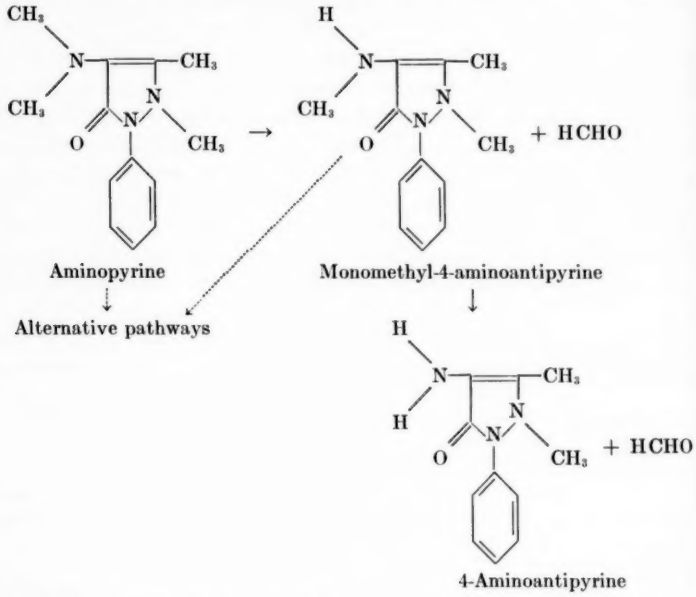
form a colored derivative. The *p*-hydroxydiphenyl derivative formed with the aldehyde from monoethyl-4-aminoantipyrine had an absorption spectrum identical with that formed with acetaldehyde. Balance studies showed that acetaldehyde and 4-aminoantipyrine were formed in equivalent amounts (0.35 μmole).

DISCUSSION

Experiments described in this paper indicate that there is an enzyme system in liver which catalyzes the oxidative demethylation of aminopyrine as outlined by the accompanying equations. This pathway postulates that the methyl groups are removed from aminopyrine in a stepwise fashion. It has not been possible to show this directly, since MMAP, the monomethyl derivative, is demethylated considerably more rapidly than is aminopyrine.

The dealkylation enzyme preparation has several unusual features: it requires both TPNH and oxygen; it is unusually non-specific; and it is localized in liver microsomes.

A requirement for both TPNH and oxygen was previously reported for the enzyme system which deaminates amphetamine (20). The requirement for both of these factors is difficult to explain. The possibility is being explored that one enzyme component in the system is a flavoprotein and that hydrogen peroxide formed in the process of oxidation of TPNH may be utilized in the dealkylation reaction.



The wide range of foreign compounds dealkylated by the microsomal enzyme preparation is indicated by work now in progress. Other compounds dealkylated by liver microsomes include quinacrine (Atabrine), meperidine (Demerol), *N*-dibenzyl-β-chloroethylamine (Dibenamine), methylamphetamine, methylaniline, and ethylaniline. However, epinephrine is not demethylated by the microsomal preparation. It is not yet known whether one or several systems are involved in the demethylation of various methylated alkylamines, nor even whether the same enzymatic mechanism is capable of removing methyl, ethyl, and butyl groups. The enzyme system in rat liver described by Mueller and Miller (14), which demethylates 4-dimethylaminoazobenzene and related dyes, is similar in some respects to the system which demethylates aminopyrine. Under Mueller and Miller's experimental conditions, however, both DPN and TPN are required for the demethylation of the dyes, and it has not been shown that the active cofactor is TPNH.

The nature of the normal substrate for the dealkylation enzyme system remains an open question. If there is a function other than the metabolism of foreign compounds, it is most unusual that such a wide range of alkylamines is attacked. In an attempt to find a normally occurring substrate, the influence of the preparation on a number of methyl compounds normally found in the body is now under investigation. In this respect, it is of interest that enzyme systems which demethylate sarcosine and dimethylglycine have been reported by Mackenzie *et al.* (21) to be located in liver mitochondria.

Recent work has shown that diethylaminoethyl diphenylpropylacetate (SKF 525-A) markedly prolongs the action of a variety of drugs *in vivo* by blocking their rate of biotransformation (18). Among the metabolic pathways inhibited by SKF 525-A both *in vivo* and in liver homogenates are side chain oxidation (*e.g.*, barbiturates), ether cleavage (*e.g.*, codeine), deamination (*e.g.*, amphetamine), and dealkylation (*e.g.*, aminopyrine and ephedrine) (19). These findings suggested that a diversity of drug metabolic pathways possessed a number of factors in common. Studies in this laboratory have shown that the systems which oxidize barbiturates (22), deaminate amphetamine (20), and cleave ethers[5] also require TPNH and oxygen and are localized in microsomes. Recent studies from this laboratory (23) indicate that the enzyme system which hydroxylates aniline, acetanilide, and quinoline is also located in liver microsomes and requires oxygen and TPNH.

SUMMARY

Aminopyrine (dimethyl-4-aminoantipyrine) and its ethyl and butyl homologues are dealkylated in rabbit, rat, and guinea pig liver homogenates to yield 4-aminoantipyrine. The methyl groups of aminopyrine and monomethyl-4-aminoantipyrine are converted to formaldehyde, and the ethyl group of the monoethyl homologue yields acetaldehyde. Both TPNH and oxygen are required, and the dealkylation system is located in the microsomes.

Diethylaminoethyl diphenylpropylacetate (SKF 525-A) inhibits the dealkylation of aminopyrine and monomethyl-4-aminoantipyrine. This inhibitor also affects the metabolism of a diversity of other types of drug enzyme systems which are located in microsomes and require TPNH and oxygen.

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THE ENZYMATIC DEAMINATION OF AMPHETAMINE (BENZEDRINE)*

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A previous study on the metabolism of amphetamine (1-phenyl-2-aminopropane), widely used as a stimulant of the central nervous system, showed that its major route of biotransformation in the rat and dog involved hydroxylation of the aromatic ring. In the rabbit, however, the compound was not hydroxylated, but was found to be transformed in another manner (2).

The present report describes an enzyme system in the rabbit which deaminates amphetamine to yield phenylacetone. It will be shown that the enzyme system is localized in the microsomal fraction of the cell and requires oxygen and reduced triphosphopyridine nucleotide. Furthermore, these studies indicate that there are inhibitory factors present in the liver of the dog, rat, and guinea pig which may explain, in part, the inability of these species to deaminate amphetamine.

Materials—*l*- and *d*-amphetamine sulfate was obtained through the courtesy of the Smith, Kline and French Laboratories. *l*-Norephedrine hydrochloride was kindly supplied by K. H. Beyer of Sharpe and Dohme. Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) 80 per cent purity, glucose-6-phosphate dehydrogenase, and glucose-6-phosphate were obtained from the Sigma Chemical Company. Reduced triphosphopyridine nucleotide (TPNH) was prepared by the procedure of Kaplan *et al.* (3).

Methods—Amphetamine, ephedrine, norephedrine, and methylamphetamine were determined by the methyl orange reaction of Brodie and Udenfriend (4) with benzene as the extractant, as previously described (2, 5). An essentially similar procedure was used for the estimation of 1-phenyl-1-aminopropane, 1-phenyl-3-aminobutane, phenylethylamine, isoamylamine, 2-aminoheptane, and benzylamine with chloroform as the extractant. Tyramine was estimated according to Udenfriend and Cooper (6) and *p*-

* A preliminary report of this work has appeared (1). The material in this investigation will appear in a thesis in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology, Graduate Council, George Washington University.

hydroxyamphetamine according to Axelrod (2). Ammonia was estimated by the Conway microdiffusion procedure (7).

Phenylacetone was determined by its reaction with 2,4-dinitrophenylhydrazine as follows: An aliquot of biological material was transferred to a 40 ml. glass-stoppered centrifuge tube containing 0.5 ml. of 1 N NaOH and 20 ml. of carbon tetrachloride (reagent grade) and shaken for 20 minutes. The tube was centrifuged and the aqueous phase removed by aspiration. The carbon tetrachloride extract was washed with 5 ml. of water to remove "blank" material which reacted with dinitrophenylhydrazine. 15 ml. of the carbon tetrachloride phase were transferred to a 40 ml. glass-stoppered centrifuge tube containing 5 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl and shaken for 2 hours. The aqueous phase was removed by aspiration and the organic phase washed three times with 5 ml. portions of 2 N HCl to remove unchanged dinitrophenylhydrazine. 6 ml. of the carbon tetrachloride extract containing the dinitrophenylhydrazone derivative of phenylacetone were transferred to a cuvette containing 1 ml. of 10 per cent KOH in ethanol, and the optical density was read in a spectrophotometer at 430 mμ. A blank consisting of biological material carried through the procedure was set at 100 per cent transmission. Standards were prepared by adding a known amount of phenylacetone to biological material and proceeding as above. Phenylacetone added to biological materials in amounts from 0.5 to 5 μmoles are recovered with adequate precision 90 ± 6 per cent.

Preparation of Tissue Samples—The preparation of all tissue samples was carried out at 0–3°.

Male albino rabbits were stunned and exsanguinated. The livers were immediately removed and homogenized with 2 volumes of 0.1 M phosphate buffer, pH 7, with a Potter-Elvehjem type of homogenizer. The homogenates were centrifuged at 9000 × *g* for 10 minutes to remove unbroken cells, nuclei, and mitochondria, and the resulting supernatant fraction was dialyzed against 0.01 M phosphate buffer, pH 7.0, for 20 hours. The supernatant fraction could be stored for at least 2 weeks at −10° without loss of activity.

The particulate fractions of liver were prepared by differential centrifugation of 12 per cent homogenates in 0.25 M sucrose (8). The nuclear fraction was sedimented by centrifugation at 600 × *g* for 10 minutes; the mitochondria at 9000 × *g* for 10 minutes; microsomes were separated from the soluble supernatant fraction by centrifugation at 78,000 × *g* for 45 minutes. The microsomes were washed once with 0.25 M sucrose and recentrifuged. Particulate fractions were resuspended in a 0.1 M phosphate buffer, pH 7.0, to a concentration 3 times that of the original homogenate. The particulate and soluble supernatant fractions were dialyzed against 0.01 M phosphate buffer, pH 7.0, for 20 hours.

Measurement of Enzyme Activity—A typical incubation mixture was prepared as follows: In a 20 ml. beaker were placed 0.3 ml. of enzyme preparation (equivalent to 100 mg. of liver), 5 μmoles of nicotinamide, 0.1 μmole of TPN, 5 μmoles of $MgCl_2$, 0.6 μmole of *l*-amphetamine, 0.5 ml. of phosphate buffer pH 7.4 (0.2 M), and water to make a final volume of 4 ml. The mixture was incubated in a Dubnoff metabolic shaking apparatus for 2 hours at 37° in air. At the end of the incubation period an aliquot of the reaction mixture was immediately transferred to a 60 ml. glass-stoppered bottle containing 0.5 ml. of 1 N NaOH and assayed for amphetamine. Enzyme activity was expressed as micromoles of amphetamine metabolized.

After the incubation the residual amphetamine was identified by comparing its distribution ratios between buffers of various pH values and benzene with those of authentic amphetamine. The results indicated that the apparent amphetamine measured after incubation had the same solubility characteristics as authentic amphetamine and that the compounds were presumably the same.

Results

Tissue Distribution of Enzyme Activity—*l*-Amphetamine (0.6 μmole) was incubated with 100 mg. of minced liver, lung, diaphragm, muscle, kidney, and brain of the rabbit under the conditions described under "Methods." The liver metabolized 0.35 μmole of *l*-amphetamine in 2 hours, while the other tissues were unable to metabolize the drug.

Properties of Enzyme System—An undialyzed supernatant fraction of rabbit liver failed to metabolize *l*-amphetamine in appreciable amounts without the addition of nicotinamide and either TPN or DPN. Nicotinamide presumably served to protect the pyridine nucleotides against enzymatic destruction (9). The effect of the two pyridine nucleotides was then examined in a supernatant fraction of rabbit liver dialyzed for 20 hours. It was found that the activity of the dialyzed supernatant fraction was restored after the addition of TPN, while DPN was inactive (Fig. 1). Mg^{++} stimulated activity about 10 per cent. Negligible activity was observed under anaerobic conditions, but incubation in air was as effective as incubation in oxygen. In phosphate buffers the maximal enzyme activity occurred at pH 7.4, and the activity was sharply reduced below and above this pH.

Rate of Metabolism—The rate of metabolism of *l*-amphetamine by the rabbit supernatant fraction is shown in Fig. 2. About 50 per cent of the drug was metabolized in 3 hours, with no further metabolism after this time. To examine the lability of the enzyme, the rabbit liver supernatant fraction was preincubated at 37° for 3 hours. On the addition of *l*-amphetamine, no metabolism of the drug took place.

The relationship between substrate concentration and substrate disappearance is shown in Table I. At concentrations of amphetamine varying from 0.6 to 2.4 μmoles a constant amount of the drug was metabolized.

Intracellular Localization of Enzyme System—Nuclear, mitochondrial, microsomal, and soluble supernatant fractions of the rabbit liver were separated by differential centrifugation (8) and assayed for their capacity to metabolize *l*-amphetamine. Negligible enzyme activity was observed in each cellular fraction (Table II). However, when the microsomal and soluble supernatant fractions were combined, the drug was metabolized

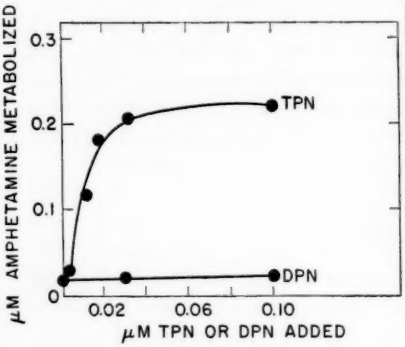


FIG. 1

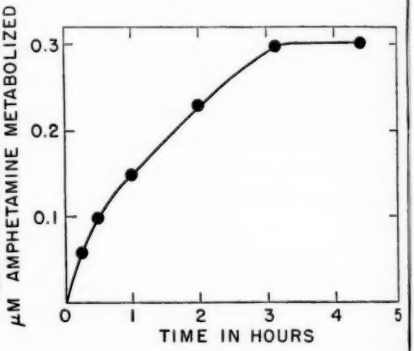


FIG. 2

FIG. 1. Requirement for TPN. 0.3 ml. of dialyzed rabbit liver supernatant fraction was incubated for 2 hours at 37° with 0.6 μmole of *l*-amphetamine, varying amounts of TPN or DPN, and cofactors described under "Methods."

FIG. 2. Rate of metabolism of *l*-amphetamine. Six beakers, each containing 0.3 ml. of rabbit liver supernatant fraction, were incubated at 37° with 0.6 μmole of *l*-amphetamine and cofactors described under "Methods."

almost as effectively as by the whole homogenate. From these observations it was evident that factors present in both the microsomes and the soluble supernatant fraction were required to carry out the metabolism of amphetamine.

Requirement for TPNH—The possible rôle of the soluble supernatant fraction in the metabolism of amphetamine was examined by adding a number of substrates normally present in this fraction to the microsomes. It was found that by replacing the soluble supernatant fluid with TPN and either glucose-6-phosphate or isocitric acid, unwashed microsomes metabolized amphetamine; washed microsomes in the presence of these factors were unable to metabolize the drug. These observations suggested that TPN-dependent dehydrogenases associated with the unwashed microsomes were involved in the enzymatic conversion of amphetamine. In

the presence of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and TPN, washed microsomes metabolized amphetamine (Table III). These results suggested that reduced TPN was the actual cofactor and that

TABLE I

Relationship between Substrate Concentration and Substrate Disappearance

0.3 ml. of rabbit liver supernatant fraction was incubated at 37° for 2 hours with various amounts of *l*-amphetamine and cofactors described under "Methods."

l-Amphetamine added	*l*-Amphetamine metabolized
μmoles	*μmole*
0.12	0.12
0.30	0.20
0.60	0.25
1.20	0.24
2.40	0.22

TABLE II

Intracellular Distribution of Amphetamine Deamination Activity

Various intracellular fractions were prepared from rabbit liver by differential centrifugation as described under "Methods." Aliquots of the various fractions equivalent to that contained in 100 mg. of whole rabbit liver were incubated at 37° for 2 hours with 0.6 μmole of *l*-amphetamine and cofactors described under "Methods."

Intracellular fraction	*l*-Amphetamine metabolized
	μmole
Whole homogenate	0.27
Nuclei*	0.06
Mitochondria	0.00
Microsomes	0.03
Soluble supernatant	0.00
Nuclei* and soluble supernatant	0.08
Mitochondria and soluble supernatant	0.02
Microsomes and soluble supernatant	0.20

* This fraction contained unbroken cells and red blood cells as well as nuclei.

TPN-dependent dehydrogenases in the soluble fraction of the cell served to generate this cofactor. To confirm this, the effect of chemically prepared TPNH on the metabolism of amphetamine by microsomes was examined. Reduced TPN was found to be as effective as the supernatant fraction in promoting the metabolism of amphetamine by the washed microsomal fraction of rabbit liver (Table III). DPNH could not replace TPNH. From these results, it appears that the supernatant fraction

served to maintain a reservoir of reduced TPN by catalyzing the oxidation of glucose-6-phosphate and other substrates and that the deamination of amphetamine was mediated by a TPNH-dependent enzyme located in the microsomal fraction of the rabbit liver.

Amphetamine Disappearance and Phenylacetone Formation—Supernatant fluid obtained from 20 gm. of rabbit liver was incubated at 37° for 1 hour with 100 μmoles of nicotinamide, 10 μmoles of TPN, 100 μmoles of $MgCl_2$, 120 μmoles of *l*-amphetamine, 10 ml. of phosphate buffer, pH 7 (0.2 M), and examined for the presence of phenylacetone, the deaminated metabolite of amphetamine. Apparent phenylacetone formed in the enzymatic

TABLE III

Requirement for Reduced TPN

0.3 ml. of liver microsomal fraction, 0.6 μmole of *l*-amphetamine, 5 μmoles of $MgCl_2$, 5 μmoles of nicotinamide, 0.5 ml. of 0.2 M phosphate buffer, pH 7.4, added cofactors, and water to make a final volume of 4 ml. were incubated at 37° for 2 hours.

Cofactors added	*l*-Amphetamine metabolized
	μmole
TPN 1 μmole*	0.01
" 0.1 μmole, glucose-6-phosphate 5 μmoles	0.03
" 0.1 " " 5 " glucose-6-phosphate dehydrogenase 1 mg.	0.25
TPNH 1 μmole*	0.26
DPNH 1 "	0.03
Soluble supernatant from 100 mg. of rabbit liver + TPN 0.1 μmole	0.26

* These cofactors were added in five divided portions over a period of 90 minutes.

deamination of amphetamine was extracted from the incubation mixture with carbon tetrachloride and treated with 2,4-dinitrophenylhydrazine as described under "Methods." The absorption spectra of the hydrazone of the enzymatically formed phenylacetone and that of the hydrazone of an authentic sample of phenylacetone were identical. Further evidence for the identity of the hydrazone of phenylacetone was obtained by ascending paper chromatography with water, *t*-butanol, and *n*-butanol (6:6:5) as the solvent system. The apparent and authentic hydrazones of phenylacetone were found to have the same R_F (0.70).

The quantity of phenylacetone in the enzymatic deamination of *l*-amphetamine was equivalent to only about one-half that of the *l*-amphetamine metabolized (Table IV). However, phenylacetone incubated with rabbit microsomes and TPNH was also metabolized. On incubation of 0.5, 1, and 2 μmoles of phenylacetone with rabbit liver microsomes, about

the same percentage of the phenylacetone disappeared. It was possible, therefore, to make an approximate correction for the metabolism of phenylacetone formed in the enzymatic deamination of *l*-amphetamine. The re-

TABLE IV

Enzymatic Deamination of l-Amphetamine to Phenylacetone

Each beaker containing 1.5 ml. of rabbit liver microsomal fraction, 25 μmoles of $MgCl_2$, 25 μmoles of nicotinamide, 2 ml. of phosphate buffer, pH 7.4 (0.2 M), added cofactors, substrates, and water to make a final volume of 10 ml. was incubated at 37° for 2 hours. Experiments 1 and 1A contained 3 μmoles of TPNH added in five divided portions over a period of 90 minutes. Experiments 2 and 2A contained 10 μmoles of glucose-6-phosphate, 1 mg. of glucose-6-phosphate dehydrogenase, and 0.3 μmole of TPN.

Experiment No.	Substrate added	Amphetamine metabolized	Phenylacetone found	Phenylacetone metabolized	Calculated amount of phenylacetone formed
		μmoles	*μmole*	*per cent*	*μmoles*
1	*l*-Amphetamine 4 μmoles	1.26	0.63		1.21
1A	Phenylacetone 1.0 μmole			48	
2	*l*-Amphetamine 4 μmoles	1.10	0.66		1.02
2A	Phenylacetone 1.0 μmole			35	

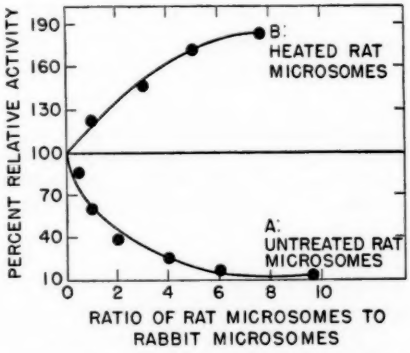


FIG. 3. Inhibition and activation of amphetamine-deaminating activity of rabbit liver microsomes by rat liver microsomes. *A*, each beaker contained 0.6 μmole of *l*-amphetamine, 0.1 μmole of TPN, 5 μmoles of $MgCl_2$, 5 μmoles of nicotinamide, 0.5 ml. of phosphate buffer, pH 7.4 (0.2 M), microsomal fraction and soluble supernatant fraction obtained from 100 mg. of rabbit liver, and varying amounts of microsomes obtained from rat liver. The reaction mixture was incubated at 37° for 2 hours. *B*, same as *A*, but rat microsomes were preheated for 2 minutes in a boiling water bath.

sults shown in Table IV indicate that about 1 mole of phenylacetone was formed for each mole of amphetamine metabolized.

From 50 to 100 per cent of the theoretical amount of ammonia was found to be liberated in the enzymatic deamination of amphetamine, but,

TABLE V

Substrate Specificity

0.3 ml. of rabbit liver supernatant fraction was incubated at 37° for 2 hours with 0.6 μmole of substrate and cofactors as described under "Methods." The reaction mixture was examined for the amount of substrate remaining. In the absence of TPN none of the substrates were metabolized.

Substrate	Relative activity
	per cent
l-Amphetamine	100
d-Amphetamine	27
l-*p*-Hydroxyamphetamine	14
l-Ephedrine	150
d-Ephedrine	11
l-Norephedrine	70
dl-Methylamphetamine	77
dl-1-Phenyl-1-aminopropane	40
dl-1-Phenyl-3-aminobutane	70
Phenylethylamine*	30
dl-α-Phenylethylamine*	27
Tyramine*	0
Benzylamine*	0
2-Aminoheptane	20
Isoamylamine*	0

* The supernatant fluid was preincubated at 37° for 10 minutes with 10^{-4} M isopropylisonicotinylhydrazine before the addition of substrates metabolized by monoamine oxidase. At this concentration isopropylisonicotinylhydrazine completely inhibits monoamine oxidase activity (10) without effecting amphetamine-deaminating activity.

owing to the relatively large amounts of endogenous ammonia also formed by the enzyme preparations, an accurate balance study could not be made.

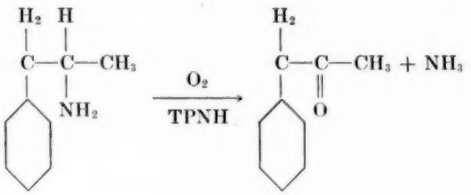
Enzyme Activity in Various Species—The activity of the amphetamine-deaminating enzyme was examined in dog, guinea pig, and rat liver supernatant fraction. Only small amounts of enzyme activity were found in these species compared to the rabbit. The possibility was entertained that the low degree of deamination activity resulted from inhibitory factors in microsomes. This was examined by measuring the effect of microsomes of dog, guinea pig, and rat liver on the enzyme activity of rabbit microsomes. The activity of the rabbit liver microsomes was markedly de-

pressed in the presence of the microsomes of these species. On the other hand, the rabbit microsomal enzyme was not inhibited by the soluble supernatant fractions of dog, guinea pig, or rat liver. These observations indicated that an inhibitory factor was present in the microsomes of dog, guinea pig, and rat liver. Fig. 3, *A* shows the inhibitory action of rat microsomes on the amphetamine-deaminating activity of rabbit liver microsomes. When the microsomal fraction of rat liver was heated at 100° for 2 minutes prior to its addition to the rabbit enzyme, a marked stimulation of enzyme activity was observed (Fig. 3, *B*). These results indicate the presence of a heat-stable activating factor which is masked by a heat-labile inhibitory factor. Preheated microsomes of dog, guinea pig, and rabbit liver also possessed stimulatory activity.

Substrate Specificity—The metabolism of a number of amines by rabbit liver supernatant fraction is recorded in Table V. Substrates having a phenylpropylamine or phenylbutylamine structure were extensively metabolized and the enzyme preparation showed relative specificity for the levo isomers. None of the amines were metabolized in the absence of TPN. It appeared that phenolic-substituted amines, phenylethylamines, and aliphatic amines were metabolized slightly or not at all. It was possible that the arylamines could also be metabolized by hydroxylation of the aromatic nucleus. However, it seemed unlikely that the phenylisopropylamines were hydroxylated in the rabbit, since it was demonstrated that this species could not hydroxylate these amines *in vivo* (2, 5). It seemed likely that the rabbit liver microsomal enzyme system metabolized the amphetamine analogues by deamination.

DISCUSSION

On the basis of the studies described in this paper, the over-all reaction for the enzymatic deamination of amphetamine is represented in the accompanying scheme. Amphetamine is deaminated to phenylacetone and ammonia in the presence of reduced TPN and oxygen by an enzyme system which is localized in the microsomal fraction of the rabbit liver. A reservoir of reduced coenzyme is maintained by TPN-dependent dehydrogenases and their oxidizable substrates present in the soluble supernatant fraction.



The enzyme which deaminates amphetamine differs from other deaminating enzymes such as monoamine oxidase, D-amino acid oxidase, L-amino acid oxidase, and glutamic acid dehydrogenase with respect to its substrate specificity, cellular localization, and cofactor requirements.

The rôle of reduced TPN in an enzyme system catalyzing the oxidative deamination of an amine is not understood. Reduced TPN could conceivably act by the generation of hydrogen peroxide through the transfer of its hydrogen by an intermediate electron transport system to molecular oxygen. Preliminary studies indicate that hydrogen peroxide generated from D-amino acid oxidase was unable to replace TPNH in the deamination of amphetamine by rabbit liver microsomes. The activating action of heated rat microsomes suggests the requirement for other factors besides TPNH. The nature of the additional cofactor is unknown.

Previous studies have shown considerable species differences in the metabolism of amphetamine *in vivo* (2). The dog and the rat transformed amphetamine mainly by hydroxylation, while the rabbit metabolized the drug, presumably by deamination. The species differences in the metabolism of amphetamine may be explained in part by the presence of inhibitory factors in the dog and rat. Inhibitory factors in these species may act by blocking the deamination of amphetamine so that the drug is excreted unchanged or metabolized by alternative pathways involving hydroxylation.

It is becoming increasingly evident that enzymes in liver microsomes which have a specific requirement for reduced TPN and oxygen are of major importance in the detoxification of many drugs and foreign organic compounds. Such enzyme systems have been found to carry out a variety of reactions such as dealkylation of alkylamines (11, 12), side chain oxidation of barbiturates,[1] cleavage of aromatic ethers,[2] and hydroxylation of aromatic compounds (13). Another property common to these enzyme systems is their inhibition by β-diethylaminoethyl diphenylpropylacetate (14, 15).

SUMMARY

An enzyme system in rabbit liver microsomes catalyzes the deamination of amphetamine to yield phenylacetone and ammonia. The enzyme system requires reduced triphosphopyridine nucleotide and oxygen. The TPN-dependent dehydrogenases in the soluble supernatant fraction of the liver serve to maintain a reservoir of reduced triphosphopyridine nucleotide.

Species differences in the metabolism of amphetamine may be explained, in part, by the presence of inhibitory factors in the microsomes of the dog

[1] Cooper, J. R., and Brodie, B. B., unpublished findings.

[2] Axelrod, J., unpublished work.

and rat liver. A heat-stable factor which can stimulate the enzymatic deamination of amphetamine is also present in the microsomes.

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SYNTHESIS OF *N*-SUBSTITUTED GLUCOSAMINES AND THEIR EFFECT ON HEXOKINASE*

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The enzyme hexokinase prepares carbohydrates for degradation by both the glycolytic and the direct oxidative pathways. For this reason, hormonal control of this enzyme in animals (1, 2) exerts a profound influence on their carbohydrate metabolism. Because synthetic inhibitors of hexokinase have proved useful in kinetic studies (3), in identifying different hexokinases (4), and in elucidating the mechanism by which glyceraldehyde inhibits glycolysis (5), we have undertaken the synthesis of a new series of inhibitors of hexokinase in the hope that kinetic studies with these compounds might aid in elucidating the mode of action of the naturally occurring inhibitory or regulatory agents (6, 7).

Harpur and Quastel (8) found that glucosamine is phosphorylated by brain hexokinase and suggested that it might possess antitumor activity because of its ability to deplete the adenosine triphosphate (ATP) supply (9). Since glucosamine competitively inhibits glucose phosphorylation (8), it seemed that the antitumor activity preliminarily reported (9) might have resulted from a decreased supply of utilizable carbohydrate rather than from a decreased supply of ATP. Better inhibitors of hexokinase were therefore sought and have been found in the form of *N*-substituted derivatives of glucosamine. As shown by Harpur and Quastel (8), *N*-acetylglucosamine is a competitive inhibitor of brain hexokinase, but, unlike free glucosamine, this derivative is not phosphorylated. Most of the compounds prepared here have been shown to behave in a manner analogous to *N*-acetylglucosamine, except for *N*-anisylideneglucosamine, which was found to be a non-competitive inhibitor of brain hexokinase. Of the compounds tested, none inhibited sarcoma 180 in mice.

Synthesis of Glucosamine Derivatives

Two alternative routes are available for the synthesis of *N*-acyl derivatives of glucosamine. The most direct method is the reaction of an acyl

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chloride with free glucosamine in an alkaline medium (10). This method proved successful for the synthesis of most of the compounds described in this paper, but it was found essential to purify the commercial glucosamine by the procedures of Breuer (11) and Westphal and Holzmann (12).

A second procedure for the synthesis of *N*-acyl derivatives of glucosamine is that of Bergmann and Zervas (13), who synthesized benzoylglucosamine from the tetra-*O*-acetyl derivative of glucosamine. Their method locates the aroyl group specifically, but suffers the disadvantage of a small yield (30 per cent) in the removal of the *O*-acetyl groups with NaOH.

Other deacetylation methods which might improve the yield were therefore sought. The catalytic barium methoxide method was found to give yields of 40 to 55 per cent. When this method was used, the anhydrous methanolic solution of the compound and barium methoxide was kept in the refrigerator for 24 hours, although deacetylation apparently proceeded as rapidly as the acetylated derivative was brought into solution. If desired, the barium may be removed with the aid of the cation exchange resin, IR-100-H (Rohm and Haas). This is usually not necessary, since the small quantity of barium present is removed in the process of purification by recrystallization.

Deacetylation with ammoniated methanol (prepared by passing anhydrous ammonia into anhydrous methanol) was found to be even more satisfactory. By this procedure the yields of deacetylated product averaged between 50 and 60 per cent.

The preferred method for obtaining the aminobenzoylglucosamine derivatives was that of hydrogenating the deacetylated nitro derivatives in hot ethanol with PtO_2 as the catalyst. Larger quantities of the acetylated nitro compounds could be handled at one time because of their greater solubility in ethanol, but it was found that, upon deacetylation of the resultant aminobenzoyl compounds, highly colored products were obtained. Only typical preparations by each of the two procedures and a typical reduction of a nitrobenzoyl derivative to the corresponding amino derivative will be described in detail. The other compounds were prepared in the same manner with only minor modifications in the solvent composition for recrystallizing. The analytical data, melting points, and rotations for the new compounds reported here are described in Table I.

Absolute ethanol and methanol were prepared by the procedure of Lund and Bjerrum (14). The acid chlorides used were Eastman, white label grade, and melted with 1–2° of the literature values.

*Tetra-O-acetyl-N-(p-nitrobenzoyl)-*D*-glucosamine*—A solution of 14.5 gm. of tetra-*O*-acetyl-D-glucosamine in 100 ml. of dry pyridine (distilled from BaO) was chilled to 0° and 8.1 gm. of *p*-nitrobenzoyl chloride were added. The solution was kept at 0° for 2 hours before pouring into 1 liter of rapidly

TABLE I
N-Substituted Glucosamine Derivatives

TABLE I

N-Substituted Glucosamine Derivatives

Compound	Mol. wt.	Formula	Per cent C		Per cent H		Per cent N		M.p.	$[\alpha]_D^{25}$
			Theory	Found	Theory	Found	Theory	Found		
									°C.	*degrees*
N-(*m*-Bromobenzoyl)glucosamine	363.2	$C_{13}H_{17}O_6NBr$*	43.0	43.4	4.72	4.44	3.86	3.96	221–222†	
N-(Phenylacetyl)glucosamine	297.3	$C_{14}H_{19}O_6N$	56.6	56.9	6.44	6.58	4.71	4.67	208–210†	41.1 ± 1.0, *C* 1.06, H_2O
N-(Phenylpropionyl)glucosamine	311.3	$C_{15}H_{21}O_6N$	57.9	58.0	6.80	6.95	4.50	4.60	216–218†	
N-(*p*-Nitrobenzoyl)tetraacetylglucosamine	496.4	$C_{21}H_{24}O_{12}N_2$	51.0	50.8	5.12	4.88	5.65	5.60	208–210	53.6 ± 0.4, *C* 5.68, $CHCl_3$
N-(*p*-Nitrobenzoyl)glucosamine	328.2	$C_{13}H_{16}O_8N_2$	47.6		4.91		8.53			
	(337.2)‡	(+0.5H_2O)	(46.3)	46.4	(5.04)	5.16	(8.31)	8.38	201–202†	53.7 ± 1.3, *C* 1.03, H_2O
N-(*m*-Nitrobenzoyl)tetraacetylglucosamine	496.4	$C_{21}H_{24}O_{12}N_2$	51.0	50.9	5.12	4.93	5.65	5.55	195–196	56.3 ± 1.0, *C* 2.68, $CHCl_3$
N-(*m*-Nitrobenzoyl)glucosamine	328.2	$C_{13}H_{16}O_8N_2$	47.6	47.4	4.91	5.11	8.53	8.60	215–216†	48.3 ± 1.3, *C* 0.476, H_2O
N-(3,5-Dinitrobenzoyl)tetraacetylglucosamine	481.4	$C_{21}H_{23}O_{14}N_3$	52.4	51.9	5.65	5.74	8.72	8.66	217	
N-(3,5-Dinitrobenzoyl)glucosamine	373.2	$C_{13}H_{15}O_{10}N_3$	41.8		4.05		11.3			
	(391.3)‡	(+ H_2O)	(39.9)	40.3	(4.37)	4.57	(10.7)	10.6	200†	45.5 ± 1.3, *C* 1.10, H_2O
N-(*p*-Aminobenzoyl)tetraacetylglucosamine	466.4	$C_{21}H_{26}O_{10}N_2$	54.1	53.8	5.62	5.77	6.00	6.09	206–207†	
N-(*p*-Aminobenzoyl)glucosamine	298.2	$C_{13}H_{18}O_8N_2$	52.4	52.6	6.07	6.22	9.39	9.22	§	
N-(*m*-Aminobenzoyl)tetraacetylglucosamine	466.4	$C_{21}H_{26}O_{10}N_2$	54.1	53.7	5.62	5.62	6.00	6.12	195–196†	
N-(*m*-Aminobenzoyl)glucosamine	298.2	$C_{13}H_{18}O_8N_2$	52.4	52.7	6.07	6.32	9.39	9.12	‖	50.0 ± 1.2, *C* 1.20, H_2O
N-(3,5-Diaminobenzoyl)tetraacetylglucosamine	481.4	$C_{21}H_{27}O_{10}N_3$	52.4	51.9	5.65	5.74	8.72	8.66	183–184†	

* Bromine, theory 22.00 per cent; found, 21.64. † Decomposition. ‡ The water of crystallization could be removed by drying at 56° *in vacuo*, but some decomposition of the anhydrous compounds resulted, as was evidenced by discoloration; figures in parentheses. § This compound begins to darken at 220° and gradually decomposes as the temperature increases. ‖ Becomes dark at 178° and gradually decomposes as the temperature increases.

stirred ice water. The product solidified; it was collected by filtration, washed with water and cold ethanol, and dried; yield, 17 gm. The analytically pure compound was obtained as long, white, thread-like crystals by repeated crystallization from absolute ethanol.

N-(*p*-*Nitrobenzoyl*)-D-*glucosamine*—(*a*) The tetra-*O*-acetyl-*N*-(*p*-nitrobenzoyl)glucosamine (2 gm.) was dissolved in ammoniated absolute methanol (50 ml.) and allowed to stand at room temperature overnight. The resultant red solution was concentrated *in vacuo* until crystals began to appear, and was then placed in the refrigerator for several hours before filtering; yield, 0.8 gm. Recrystallization was accomplished from 95 per cent ethanol. (*b*) Deacetylation by the catalytic barium methoxide procedure was accomplished by dissolving 5.4 gm. of tetra-*O*-acetyl-*N*-(*p*-nitrobenzoyl)glucosamine in 50 ml. of absolute methanol. 1 ml. of 2.5 N barium methoxide was then added and the solution kept in the refrigerator for 24 hours. The product was isolated from the yellow solution as described in (*a*) above; yield, 1.9 gm.

Direct Synthesis of N-(*p*-*Nitrobenzoyl*)-D-*glucosamine*—A solution of 5 gm. of glucosamine in 25 ml. of water was immersed in an ice bath and stirred vigorously while 5.4 gm. of *p*-nitrobenzoyl chloride were added in small portions. During the reaction, a total of 25 ml. of 1 N NaOH was slowly added so as to keep the pH of the solution between 8 and 9.[1] The temperature of the reaction mixture was kept below 5° at all times, and stirring was continued for 2 hours after the final addition. The mixture was then neutralized with HCl and, while still cold, the product was collected by filtration. It was purified by two recrystallizations from 95 per cent ethanol; yield, 3.0 gm.

N-(*p*-*Aminobenzoyl*)-D-*glucosamine*—A solution of 0.53 gm. of *N*-(*p*-nitrobenzoyl)glucosamine in about 50 ml. of hot 80 per cent ethanol was shaken with 0.05 gm. of PtO_2 in an atmosphere of H_2. The initial rate of hydrogen consumption was very rapid. When the theoretical amount of H_2 for reduction of the nitro group had been consumed (5 to 15 minutes were required), the catalyst was filtered off. Crystals began to appear as the solution cooled. After the mixture had been kept at −5° to complete crystallization, the product was collected by filtration and dried *in vacuo*. A small additional quantity (12 mg.) was recovered by concentrating the filtrate *in vacuo* and crystallizing again at −5°. Yield, 0.324 gm.; the compound darkened when heated to 220°, but did not melt below 300°.

Inhibition of Hexokinase by Glucosamine Derivatives

The synthetic derivatives of glucosamine were tested as inhibitors of both brain and yeast hexokinase. An aqueous extract of beef brain ace-

[1] In some of the preparations (*e.g.*, the phenylacetyl and phenylpropionyl derivatives) better yields were obtained when 1 N Na_2CO_3 was used to maintain the pH.

tone powder (6) was dialyzed and lyophilized as described elsewhere (15). When this lyophilized preparation was dissolved in redistilled water and centrifuged at 100,000 × *g* for 30 minutes, from 85 to 90 per cent of the hexokinase activity remained in the clear supernatant solution. This is in marked contrast to extracts of fresh brain in which most of the enzyme is associated with particles which sediment at much lower centrifugal force (3).

The manometric assay for hexokinase (16) was employed with conditions as described previously (15). Following a 10 minute equilibration period, ATP was tipped into the reaction mixture from a side arm. Manometer

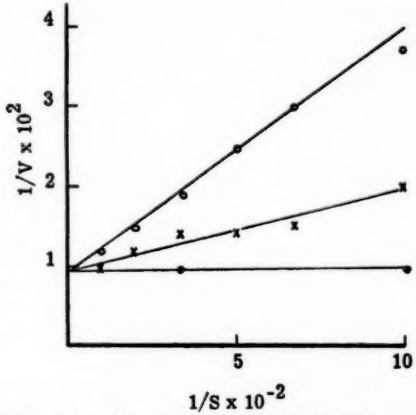

FIG. 1. Competitive inhibition of glucose phosphorylation by *N*-(3,5-dinitrobenzoyl)-D-glucosamine. ●, 0; ×, 1.3 × 10^{-4} M, and ○, 5 × 10^{-4} M *N*-(3,5-dinitrobenzoyl)glucosamine. The velocity is expressed in terms of microliters of CO_2 per 30 minutes.

readings were made 5 minutes later and at 10 minute intervals thereafter. With glucose as the substrate, the velocities were calculated from the linear portion of the rate curve. For fructose, which has a much higher K_s (15, 17), the initial velocity was estimated by extrapolating the observed values to zero time (15).

Each inhibitor was tested at two concentrations in the presence of varying concentrations (from 0.001 M to 0.01 M) of glucose and fructose. A flask containing all components except glucose or fructose was included in each experiment. None of the glucosamine derivatives tested was phosphorylated at a detectable rate. The data were plotted according to Lineweaver and Burk, and the dissociation constants of the enzyme inhibitor complexes were calculated (18). Fig. 1 depicts the inhibition, by dinitrobenzoylglucosamine, of glucose phosphorylation. Since it is difficult to

measure K_s for glucose manometrically (15), an arbitrary value of 1×10^{-4} M (8, 15) was used to calculate K_i for the various competitive inhibitors with glucose as the substrate. The results are summarized in Table II.

TABLE II

K_i Values for Competitive Inhibition of Beef Brain Hexokinase by Glucosamine Derivatives

Inhibitor	Substrate	
	Glucose	Fructose
	M	M
N-(*p*-Nitrobenzoyl)glucosamine	4×10^{-5}	5×10^{-5}
N-(*m*-Nitrobenzoyl)glucosamine	3.3×10^{-5}	8.4×10^{-6}
N-(3,5-Dinitrobenzoyl)glucosamine	1.1×10^{-5}	4×10^{-6}
N-(*p*-Aminobenzoyl)glucosamine	2×10^{-4}	1.1×10^{-4}
N-(*m*-Aminobenzoyl)glucosamine	1.5×10^{-4}	8.1×10^{-5}
N-Benzoylglucosamine		3.6×10^{-5}
N-Acetylglucosamine		4.6×10^{-4}
N-Phenylacetylglucosamine		8.6×10^{-4}

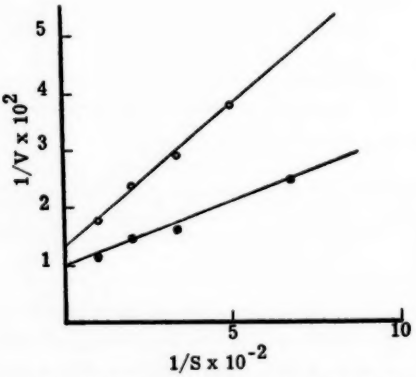

FIG. 2. Non-competitive inhibition of fructose phosphorylation by *N*-anisylideneglucosamine. ●, no inhibitor; ○, 1.7×10^{-3} M *N*-anisylideneglucosamine. The velocity is expressed in terms of microliters of CO_2 per 10 minutes.

The K_i values are only approximate because of complications in the assay. The brain hexokinase preparation contains phosphohexokinase and the CO_2 evolved results from phosphorylation by both enzymes. In the preparations of brain hexokinase tested, phosphohexokinase activity was equal to or greater than hexokinase, although this result may be more apparent than real (3, 19). Nonetheless, hexokinase was *assumed* to be the rate-

limiting enzyme in the assay. For these various reasons, no significance can be attached to the differences in K_i values for any given inhibitor with glucose and fructose, respectively, as substrates.

N-Anisylideneglucosamine[2] was found to inhibit fructose phosphorylation by brain hexokinase in a non-competitive manner with a K_i of about 0.005 M (Fig. 2).

The phosphorylation of glucose by yeast hexokinase was also inhibited by the glucosamine derivatives tested (Table III). Liver fructokinase was not significantly inhibited by 0.006 M *N*-(*p*-nitrobenzoyl)glucosamine.

TABLE III

Inhibition of Yeast Hexokinase by Glucosamine Derivatives

Compound tested	Concentration	Per cent inhibition
	M	
N-(*p*-Nitrobenzoyl)glucosamine	5×10^{-3}	24
N-(*m*-Nitrobenzoyl)glucosamine	2×10^{-3}	47
N-(3,5-Dinitrobenzoyl)glucosamine	2.5×10^{-3}	66
N-(*m*-Aminobenzoyl)glucosamine	5×10^{-3}	18
N-(*p*-Aminobenzoyl)glucosamine	4×10^{-3}	19
N-Benzoylglucosamine	5×10^{-3}	20
N-Phenylacetylglucosamine	5×10^{-3}	6
N-Acetylglucosamine	5×10^{-3}	6

The substrate was 0.005 M glucose.

Effect of Glucosamine Derivatives on Tumor-Bearing Mice[3]

Several of the compounds synthesized were tested as possible inhibitors of tumor growth in mice. The techniques employed were similar to those of Quastel and Cantero (9), except that white mice bearing Crocker sarcoma 180 were used. The compounds tested and the daily dose per mouse were as follows: glucosamine hydrochloride, 5 mg.; *N*-(*p*-aminobenzoyl)-glucosamine, 7 mg.; *N*-benzoylglucosamine, 6.6 mg.; *N*-(*meta*-nitrobenzoyl)glucosamine, 7.5 mg.; *N*-acetylglucosamine, 5 mg. In another experiment *N*-(dinitrobenzoyl)glucosamine was injected at 650 mg. per kilo of body weight each day for 5 days only. In no case was the growth of the tumors or the percentage of tumor regressions in the treated animals significantly different from that of saline-injected control mice.

[2] Dr. Robert Crane, Washington University, has tested this compound with the particulate brain hexokinase and obtained a K_i value of about 0.01 M with fructose as the substrate. He found dinitrobenzoylglucosamine to inhibit competitively with a K_i of 1×10^{-6} M.

[3] We are indebted to Dr. K. K. Chen, Dr. P. N. Harris, and Dr. J. Probst of the Lilly Research Laboratories for these assays.

DISCUSSION

The competitive nature of the inhibition of phosphorylation indicates that the substituted glucosamine compounds combine with the active enzyme site, yet none of these compounds appeared to be phosphorylated, whereas free glucosamine is. Molecular models indicate that the substituted group does not overlap the 6 position of the sugar, and, therefore, other reasons must be sought to explain their failure to become phosphorylated. One possibility is that the substituent attached to the 2-amino position interferes with the ability of hexokinase to bind ATP. It appears that the intrinsic nature of the enzyme is such that the *meta*-substituted nitrobenzoyl derivatives of glucosamine are more effectively bound than the *para*-nitrobenzoyl or benzoyl derivatives, with two nitro groups being 2 to 3 times as potent as one.

The *N*-acyl derivatives of glucosamine are probably hydrolyzed by the various cathepsins in animal tissues, and this may be one reason for their failure to inhibit tumor growth. Other derivatives of glucosamine which are less likely to be hydrolyzed by tissue enzymes are therefore being synthesized, and their biological properties will be tested.

SUMMARY

The synthesis of the following *N*-acyl derivatives of D-glucosamine is described: *m*-bromobenzoyl-, phenylacetyl-, phenylpropionyl-, *p*-nitrobenzoyl-, *m*-nitrobenzoyl-, 3,5-dinitrobenzoyl-, *p*-aminobenzoyl-, and *m*-aminobenzoyl-D-glucosamine.

These glucosamine derivatives are powerful competitive inhibitors of beef brain hexokinase ($K_i = 10^{-3}$ to 10^{-6} M) but are not phosphorylated. *N*-Anisylideneglucosamine was found to inhibit brain hexokinase in a non-competitive manner with respect to fructose.

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THE EFFECT OF ESTROGEN ON THE PRODUCTION OF A PEROXIDASE IN THE RAT UTERUS*

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McShan and Meyer (1) observed an inhibition of the succinoxidase system of rat liver homogenate upon the addition of a small amount of diethylstilbestrol, hexestrol, and dienestrol. The locus of inhibition was considered to be cytochrome oxidase. Case and Dickens (2) amplified these observations by investigating a wider range of estrogens and chemically related compounds of slight estrogenic potency. The inhibition *in vitro* of the succinoxidase system by these compounds was found not to be correlated with estrogenic potency and the site of inhibition was not the same with the different estrogens studied.

Hochster and Quastel (3) reported that oxidized diethylstilbestrol could act as a hydrogen acceptor in certain enzyme systems. In view of these findings and since this laboratory has been engaged in the separation and study of the "individual" components of the succinoxidase system (4–7), it was of interest to investigate the various components of the succinoxidase system in the rat uterus following stimulation with estrogen.

During the course of this study, it was found that the injection of estrogens into the ovariectomized rat caused a pronounced activity of peroxidase in the uterus, whereas this activity was normally extremely low. Further, the peroxidase activity was related to the amount of estrogen injected.

Methods

Female rats of the Wistar strain, weighing from 180 to 200 gm., were ovariectomized under aseptic conditions. 4 weeks after ovariectomy the animals were injected intramuscularly with 0.2 ml. of cottonseed oil for controls or with 0.2 ml. of a cottonseed oil solution of estrogen. The amounts of diethylstilbestrol or 17β-estradiol used for injection were 0.05, 0.10, 0.20, 0.30 mg. The rats were sacrificed by decapitation at intervals of up to 4 days after injection of the hormone. The uterus of each animal was quickly removed, stripped of visible fat, and chilled. The uteri of three rats were pooled and 20 per cent homogenates in cold 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, were prepared in a chilled all-glass homogenizer.

* This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

XUM

Deoxyribonucleic acid (DNA) was determined by the method of Schneider (8) with the Dische reagent. DNA for standard solutions was prepared by the procedure of Kay, Simmons, and Dounce (9). After dialysis dry weights were determined for each preparation.

Peroxidase activity was determined by a modification of the method of Smith, Robinson, and Stotz (10). The following procedure was employed. To a test-tube ($\frac{1}{2}$ inch $\times$ 4 inches) were added 1.5 ml. of 0.2 M phosphate-citrate buffer (McIlvaine), pH 6.0, 0.1 ml. of 0.06 per cent hydrogen peroxide, 0.5 ml. of 35 mg. per cent (approximately 0.001 M) leuco dye, 0.1 ml. of appropriate dilutions of tissue homogenate, and sufficient water to make a total volume of 3.0 ml. The leuco dye was prepared by dissolving 35 mg. of 2,6-dichlorobenzenoneindo-3′-chlorophenol in 80 ml. of H_2O and 20 ml. of the 0.2 M phosphate-citrate buffer, pH 6.0. The dye was reduced by passing a stream of hydrogen through the solution in the presence of palladized asbestos according to the method of Smith and Stotz (11). The rate of leuco dye oxidation was followed in the Lumetron colorimeter. A unit of peroxidase is defined as that amount of enzyme which causes a $\Delta \log I$ per minute of 1.0 under the specified conditions of the test. Peroxidase activity is defined as units of peroxidase per ml. of solution.

Catalase was determined by minor modifications of the method described by Sumner and Somers (12).

Results

The original plan of investigation had been to study the various components of the succinoxidase system in the uteri of ovariectomized rats following injection with varying amounts of estrogen. The first series of experiments was directed toward studying the possible changes in cytochrome oxidase. At first, it appeared that there were great increases in cytochrome oxidase activity in homogenates prepared from the uteri of ovariectomized, hormone-injected rats. It was found, however, that this activity was not dependent on cytochrome *c*. Instead, the activity was abolished by addition of crystalline catalase to the test system, showing it was dependent on the presence of hydrogen peroxide. It was therefore apparent that the dye-oxidizing activity was due to a peroxidase, and apparently one not previously described as being present in the rat uterus.

Before studying the response of the uterine peroxidase to estrogen administration, preliminary experiments were designed to reveal certain properties of the enzyme which are pertinent for a valid assay method. The relation of the amount of homogenate of estrogen-stimulated uterus to the rate of the reaction studied is illustrated in Fig. 1. The linear relation obtained demonstrates that the initial rate of dye oxidation employed in the assay is directly proportional to the amount of peroxidase present in the homogenate.

The relation of hydrogen peroxide concentration to the rate of the peroxidase reaction is illustrated in Fig. 2. It is noted that the peroxide concentration in the reaction mixture for maximal rate of the reaction is 0.002

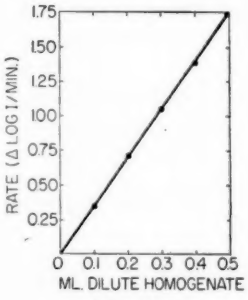

FIG. 1

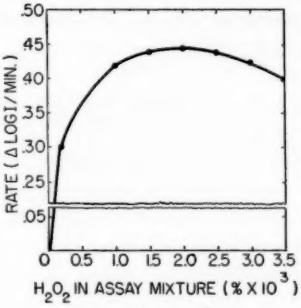

FIG. 2

FIG. 1. Relation of the amount of homogenate to the rate of the peroxidase reaction. The ovariectomized rats were injected with 0.2 mg. of diethylstilbestrol and killed 72 hours later; a 20 per cent homogenate of the uteri was prepared and diluted 1:100 with 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, for the purpose of this experiment.

FIG. 2. Relation of the hydrogen peroxide concentration to the rate of the peroxidase reaction. 0.1 ml. of a 1:100 dilution of 20 per cent homogenate, prepared from uteri of hormone-stimulated rats, was employed for each determination.

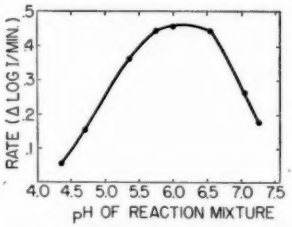

FIG. 3. The effect of pH on the peroxidase reaction. All rates were corrected for the leuco dye oxidation in the absence of the enzyme.

per cent and that the enzyme is inhibited somewhat at higher concentrations.

A study of the effect of pH, recorded in Fig. 3, shows that an optimal rate of the reaction occurs at pH 6.0. At this pH neither the rate of autoxidation of the leuco dye nor the uncatalyzed oxidation of the dye by the peroxide was significant.

Heating the homogenate at 90° for 5 minutes results in complete loss of peroxidase activity. Homogenates prepared in distilled water show a rather rapid loss of activity, while the activity of homogenates prepared

in 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, remains essentially constant for several hours in the cold. A gradual loss in activity occurs when such homogenates are frozen for several months.

Dependence of Peroxidase Activity on Estrogen—In order to evaluate the peroxidase activity of the tissue independently of the cell proliferation and other changes that occur after hormone stimulation, the activity was based on the deoxyribonucleic acid content as well as on the dry weight of the homogenates. Graded doses of both diethylstilbestrol and 17β-estradiol were injected into the ovariectomized rats, and the peroxidase, deoxyribonucleic acid, and dry weight of the uteri were determined 72 hours after injection of the hormones. The results are presented in Table I. It is clear that the peroxidase activity of the uterus of the ovariectomized rat increases from a nearly negligible amount to high levels after injection of

TABLE I

Peroxidase Levels in Uteri of Ovariectomized Rats after Injection of Diethylstilbestrol and 17β-Estradiol

Hormone injected		Peroxidase	
	mg.	*units per mg. dry weight*	*units per mg. DNA*
None		0.09	1.50
Diethylstilbestrol	0.05	2.9	60.0
"	0.10	8.9	143.0
"	0.20	18.0	300.0
"	0.30	12.7	
17β-Estradiol	0.10	8.0	
"	0.20	16.5	

Each figure is the average of three separate experiments, and the homogenate for each experiment represents the pooling of three uteri. The animals were killed 72 hours after intramuscular injection of the hormones.

TABLE II

Peroxidase Activity of Rat Uterus at Successive Intervals after Injection with Diethylstilbestrol

Time of sacrifice after injection	Peroxidase
hrs.	*units per mg. dry weight*
24	6.0
48	13.6
72	18.0
96	9.1

Each ovariectomized animal was injected with 0.2 mg. of diethylstilbestrol. Each figure is the average of three experiments, and the homogenate used in each experiment represents three pooled uteri.

the hormones. The magnitude of the effect is essentially the same for diethylstilbestrol and 17β-estradiol, and the response is a graded one for increasing amounts of diethylstilbestrol until a maximum of some 200-fold increase[1] is obtained at about 0.2 mg. The results are similar whether the peroxidase activities are based on deoxyribonucleic acid or dry weight, showing that the increase is independent of possible changes in cellularity of the tissue.

In addition to dosage, the time which elapses after injection of the hormone also determines the peroxidase response in the uterine tissue. The data in Table II indicate that a maximal response is obtained at approximately 72 hours, with a lesser response at a later interval.

Catalase Effect—Since catalase inhibits the apparent peroxidase activity in the assay system, it seemed possible that the observed peroxidase increase in the uterus after hormonal stimulation might actually be due to a decrease in catalase activity. It was shown, however, that the catalase activity was the same in unstimulated and stimulated uterine tissue, namely, a *Kat. f.* of 0.9.

DISCUSSION

The magnitude and rapidity of the response of peroxidase activity of the uterus to hormone stimulation are remarkable. Whether this represents a rapid synthesis of the enzyme or whether the hormone plays a coenzyme-like rôle is not evident from the experiments reported. In this connection it is of interest that both 17β-estradiol and diethylstilbestrol, having dissimilar chemical structures but common physiological effects, elicit similar responses.

The significance of the peroxidase response to tissue proliferation is of special interest and suggests a connection between peroxidase and the metabolism of normal and abnormal growing tissue. Since the rôle of peroxidase in animal tissue is poorly understood, the findings reported demand further study of the distribution of this enzyme and the mechanism by which it might participate in oxidative metabolism. Studies in these various directions are in progress in this laboratory.

SUMMARY

Injection of diethylstilbestrol or 17β-estradiol into ovariectomized rats causes a pronounced peroxidase activity in the uterus. The magnitude of

[1] Since the peroxidase activity of the uterus of uninjected, ovariectomized rats is so low as to be hardly measurable by the method employed, the increase upon hormone injection may actually be more than 200-fold. The low activity of the control tissue may in fact be due to the non-enzymatic peroxidase activity of the small amount of hemoglobin present in the homogenate. Such non-enzymatic peroxidase activity remains low in homogenates of the hormone-stimulated uterus.

the response is related to the amount of hormone injected and is maximal 72 hours after the injection. The significance of the findings to problems of hormone-enzyme relationships, of tissue proliferation, and of the rôle of peroxidase in animal tissue is discussed.

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METABOLISM OF COMPOUNDS RELATED TO 3-HYDROXYANTHRANILIC ACID*

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The rôle of 3-hydroxyanthranilic acid (3-OHAA) as a niacin precursor has been clearly indicated by its growth-promoting activity in the nicotinic acid (NA)-deficient rat (1–3). The over-all conversion to pyridine nucleotides must be small, since a relatively large amount is required to substitute for NA. The limited conversion of 3-OHAA to NA or its equivalent indicated that some derivative of 3-OHAA, rather than 3-OHAA itself, might be the natural substrate. Several considerations suggested the amide (3-OHAAA) as a possibility. It seemed conceivable that this amide might be metabolized by a mechanism analogous to that proposed for the acid to give nicotinic acid amide (NAA), which appears to be the more physiological form of the vitamin.

If the presence of the amide group were not advantageous for the direct conversion to pyridine derivatives, 3-OHAAA might still serve as a precursor by conversion to 3-OHAA. If this amide were hydrolyzed as is anthranilamide (AAA) in rabbits (4), it might give rise to the acid in a particular form or in a particular site well suited for further metabolism.

The possibility of utilization of anthranilic acid (AA) or its amide as a source of niacin was still attractive, despite the negative results obtained *in vivo* with the former (5). The hydroxylation of AA in the 3 position by rat liver preparations was reported by Wiss and Hellmann (6). They tentatively identified the product as the phosphate ester of 3-OHAA.

Anthranilic acid, anthranilic acid amide, and 3-hydroxyanthranilamide were investigated with regard to their effectiveness in increasing the urinary excretion of NA and quinolinic acid (QA). The ability of anthranilic acid and 3-hydroxyanthranilamide to restore the growth of niacin-deficient rats was also tested. 3-Hydroxyanthranilamide and 3-hydroxyanthranilic

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‡ Some of these data have been taken from a thesis submitted by R. E. Koski in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois.

acid methyl ester were checked as possible substrates for the 3-hydroxyanthranilic acid-oxidizing enzyme of rat liver.

EXPERIMENTAL

Test Compounds—Anthranilic acid and tryptophan were purified commercial samples. Anthranilamide was prepared by reducing *o*-nitrobenzamide with $FeSO_4$. It had a melting point of 112–113°, gave a violet, fluorescent spot with an R_F value of 0.80 when chromatographed on filter paper with the methanol-*n*-butanol-benzene-water system (2:1:1:1). 3-Hydroxyanthranilamide, which has not been previously described, was synthesized as follows.

2-Nitro-3-methoxybenzoic Acid (*I*)—In a 500 ml., 3-necked flask provided with a very effective mechanical stirrer, dropping funnel, and thermometer, 50 gm. of 3-methoxybenzoic acid were suspended in 130 ml. of acetic anhydride; the mixture was chilled and treated dropwise for 1.5 hours with a carefully prepared mixture of 15 gm. of fuming nitric acid and 20 ml. of acetic anhydride at a temperature of 2°. The suspension was held at 0–5° for 12 hours and then diluted with 400 ml. of water and filtered. The undesired isomers were removed by extracting the mixture with 2 parts of boiling ethanol. The 2-nitro derivative was then crystallized from 95 per cent ethanol to give pale greenish yellow plates (yield, 19 to 23 per cent); m.p. 261–262°, as previously reported.

2-Nitro-3-hydroxybenzoic Acid (*II*)—The demethylation was accomplished by refluxing I with acetic acid and an excess of HBr (7). For example, 8.6 gm. of I, dissolved in 110 ml. of boiling acetic acid, were treated with 60 ml. of HBr (D = 1.48) and then refluxed for 24 hours. The solution was concentrated *in vacuo* to a yellow crystalline mass which was extracted with water. The aqueous extract was filtered and carefully concentrated *in vacuo*. Sweet, yellow needles were obtained (m.p. 173–174°; yield, 35 per cent). The product was sufficiently pure for further use (reported m.p. 180.8–181.5° (8)).

2-Nitro-3-hydroxybenzamide (*III*)—1.10 gm. of II, dried at 110°, were warmed gently on a water bath with 9.9 gm. of thionyl chloride until a clear red solution was obtained (10 minutes). This solution was concentrated *in vacuo* at a temperature not exceeding 50°.[1] The acyl chloride was obtained as a red sirup, which was dissolved in 50 ml. of anhydrous chloroform; the solution was chilled to 0° and then saturated with anhydrous ammonia, with 30 minutes exposure to a slow stream of gas. The suspension obtained was extracted with 15 ml. of water. The ammonium salt of the amide dissolved in the aqueous layer, from which the amide was

[1] An explosion occurs if the nitroacyl halide is heated at higher temperature (50–100°) when $SOCl_2$ is still present, in agreement with reports for similar nitroacyl halides (9).

obtained by adding hydrochloric acid to pH 4.5 and chilling. The crystalline precipitate was practically pure 2-nitro-3-hydroxybenzamide (yield, 88 per cent). The amide crystallized from 10 volumes of hot water in long, yellow needles; m.p. 174–176°. Its solubility in 95 per cent ethanol at 27° is about 7 per cent. The infra-red analysis gave the expected absorption spectrum.

$C_7H_5O_4N_2$.	Calculated.	C 46.16, H 3.32, N 15.38
	Found.	" 46.20, " 3.16, " 15.41

3-Hydroxyanthranilamide (3-OHAAA)—0.36 gm. of III, dissolved in 10 ml. of ethanol, was reduced with hydrogen in the presence of 0.15 gm. of palladium catalyst (6 per cent on $SrCO_3$), the required amount of hydrogen being consumed in 90 minutes. The solution was filtered and taken to dryness *in vacuo*. The pink crystals were recrystallized from hot water in the presence of charcoal to give orange-yellow prisms, m.p. 157.5–159° (softening 153–156°) on the Kofler micro block, and m.p. 155.5–156.5° in a capillary tube[2] (yield, 43 per cent).

$C_7H_8O_2N_2$.	Calculated.	C 55.26, H 5.29, N 18.42
	Found.	" 55.27, " 5.38, " 18.15

The infra-red spectrum confirmed the structure.

3-Hydroxyanthranilic acid was prepared by simultaneous demethylation and reduction of I by the method of Spada and Gavioli (11) and purified according to Hegedüs (12).

Growth Studies—Young albino rats were obtained from the departmental colony, and litter mates were distributed in groups as evenly as possible. The rats were allowed to reach a weight of about 45 gm. and then fed *ad libitum* the nicotinic acid-free diet containing 0.1 to 0.2 per cent of added DL-threonine (13). When the growth had ceased, the test compounds were administered in 0.8 M glucose or 0.9 per cent NaCl solution by intraperitoneal injection. The solutions or suspensions were adjusted to pH 6 to 7; 0.5 to 1 ml., containing 0.025 mmole, was injected once each day. Rats given a higher dosage (0.05 mmole) received two injections per day, 6 hours apart. The compounds were injected daily over a period of 9 days or 21 days in different experiments, and body weights were recorded for 3 weeks. In Table I is shown the total average growth for each group of animals at the end of the period of administration. Neither anthranilic acid nor 3-hydroxyanthranilamide restored the growth of the deficient animals, while L-tryptophan and 3-hydroxyanthranilic acid were effective.

[2] The same compound was obtained in less satisfactory yield by ammonolysis of the methyl ester of 3-OHAA in a sealed tube at 115° for 2 hours. The methyl ester was prepared by refluxing 3-OHAA with H_2SO_4 in anhydrous CH_3OH and recrystallized from benzene; the melting point and analysis agree with the reports of Musajo *et al.* (10).

To exclude any toxic effect of AA, L-tryptophan was administered to half of the animals still receiving AA. The deficiency symptoms disappeared and a marked increase of growth occurred within 2 days. No growth studies were made with AAA since the lack of conversion to pyridine derivatives reported below indicated that this compound, like AA, was not a precursor of nicotinic acid.

In Vitro Studies—2 μmoles each of 3-OHAAA, 3-OHAA, or its methyl ester were incubated in Warburg flasks with 25 mg. of beef liver acetone powder extract in the presence of 10^{-3} M ferrous iron in an air atmosphere.

TABLE I

Replacement of Niacin for Growth of Rats

No. of rats	Injected	Daily dose	Total average growth
	Experiment 1, 21 days duration; female rats		
		mmole	*gm.*
2	L-Tryptophan	0.025	33.5
3	Anthranilic acid	0.025	6.6
3	" "	0.05	5.0
3	0.8 M glucose		4.6
	Experiment 2, 9 days duration; male rats		
3	L-Tryptophan	0.05	40.6
4	3-Hydroxyanthranilic acid	0.05	37.0
4	3-Hydroxyanthranilamide	0.05	2.25
4	0.9% NaCl		1.75

The oxygen uptake and the formation of quinolinic acid are shown in Table II.

It is evident that the enzyme system which metabolizes 3-OHAA to QA does not act on the amide. The data obtained for the methyl ester indicate that it is partially oxidized apparently by a different pathway, since very small amounts of QA were formed in comparison with the control flask containing 3-OHAA.[3]

Excretion Studies—Growing or young adult male rats receiving the same nicotinic acid-free diet fed in the growth experiments were used to study the urinary excretion of NA and QA, following the administration of the test compounds. AA was fed by stomach tube, in aqueous solution, at a level of 200 mg. per rat per day in three equal doses, or was injected intraperitoneally in 0.9 per cent saline solution at a level of 169 mg. per day in four

[3] We are indebted to Dr. Calvin L. Long for assistance with these experiments.

doses 3 hours apart. AAA was fed by tube at a daily level of 200 mg.; 3-OHAAA was injected intraperitoneally at a level of 7.6 mg. per day (0.05 mM). The excretion data reported for this compound were obtained during the growth studies described above.

A 24 or 48 hour control urine collection was usually made the day before the compounds were given. The 24 hour experimental collection was

TABLE II

Oxidation of 3-Hydroxyanthranilic Acid and Derivatives by Beef Liver Acetone Powder Extracts

Substrate	Oxygen uptake		QA formed in
	30 min.	60 min.	60 min.
	μmoles	*μmoles*	*μmoles*
3-OHAAA	0.138	0.075	0.00
3-OHAA methyl ester	0.59	0.85	0.18
"	1.89	1.95	1.67

TABLE III

Conversion of Administered Compounds to Urinary Quinolinic Acid

No. of rats	Weight	Compound	Daily dose	Route	Average per cent conversion			
					1st day	2nd day	3rd day	7th day
	gm.		*mg.*					
5	130–200	AA	200	*Per os**	0.08			
4	310–370	"	200	" "	0.51	0.45	0.35	
4	310–370	"	169	Intraperitoneal	0.26			
7	135–235	AAA	200	*Per os**	0			
4	40–80	3-OHAAA	7.6	Intraperitoneal	0			0.09
4	52–74	LT	10.2	"	5			5.6
4	49–75	3-OHAA	7.6	"	1.6			7

* By stomach tube.

started at the time of the first dose. All urine samples were collected under toluene and analyzed for nicotinic acid and quinolinic acid by using the *Lactobacillus arabinosus* 17-5 differential assay (3).

The results are summarized in Table III. The percentage conversion is calculated from the increase of QA over that excreted in the control period. The data indicate that AA is metabolized to QA to a slight extent, while AAA and 3-OHAAA do not give rise to a measurable increase in the QA excretion. These results are in agreement with the lack of growth reported above for AA and 3-OHAAA.

In view of the report of Bray *et al.* (4) that the rabbit hydroxylates AAA but not AA, it seemed to be of interest to ascertain whether the rat could accomplish these hydroxylations and whether the 3-OHAAA or any other product arising from these compounds could serve as precursors of QA. Unhydrolyzed urine from rats and rabbits which had received AA and AAA was given by stomach tube to rats. No increase in QA excretion resulted. Portions of the same urine samples, hydrolyzed by refluxing with 3 N HCl for 4 hours, gave rise to increased QA excretion by the rat when AAA, but not AA, was the compound originally administered. The increase was very small, indicating 0.04 to 0.1 per cent conversion of the amide administered. This may reflect oxidation of the AAA or simply extensive excretion of AAA unchanged. In the latter case, AA would arise by hydrolysis and be partially converted to QA.

DISCUSSION

In agreement with previous reports (*e.g.* Krehl *et al.* (5)), anthranilic acid was unable to replace niacin for the growth of rats. It seems likely that very little oxidation of AA in the 3 position occurs in rats. Shirai (14), using rabbits, and Kotake (15), with rats, found only 5-OHAA in the urine, while Bray *et al.* (4) could identify no hydroxylated products after administration of AA to rabbits.

The fact that AAA did not give rise directly to urinary quinolinic acid, while AA did to a slight extent, suggests that the hydrolysis of the amide, which Bray *et al.* (4) estimated at 12 to 33 per cent in the rabbit, was in our experiments insufficient to result in the formation of enough AA to be detected by QA excretions.

The results of experiments *in vivo* with 3-OHAAA reported here give evidence that the rat is unable to hydrolyze the amide linkage of the hydroxylated amide. This is in agreement with the data concerning the stability of the amide linkage in hydroxylated aromatic carboxamides in rabbits (16). The formation of 3-OHAA would have been manifested in restored growth and increased excretion of urinary pyridine derivatives, as was observed in 3-OHAA-treated control animals.

SUMMARY

1. 3-Hydroxyanthranilamide was synthesized and found to possess no niacin-replacing activity for the rat. It will not serve as a substrate for the 3-hydroxyanthranilic acid-oxidizing enzyme of liver. Thus the amide is not converted directly or by hydrolysis through 3-hydroxyanthranilic acid to pyridine derivatives.

2. Slight hydroxylation of anthranilic acid and, possibly, of its amide by the rat was indicated by results of excretion studies. The hydroxylation

was insufficient to allow any demonstrable niacin-replacing activity in growth studies.

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STUDIES IN VITAMIN E DEFICIENCY

I. THE OXYGEN CONSUMPTION OF VARIOUS TISSUES FROM THE RABBIT*

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It has been firmly established that a remarkable increase in oxygen consumption occurs in the skeletal muscle of animals with nutritional muscular dystrophy (1–9). The purpose of the present study was to ascertain whether other tissues from vitamin E-deprived rabbits also responded with an increased respiration.[1] In 1938 Telford *et al.* (10) reported no change in the rate of respiration of thyroid glands in vitamin E deficiency, and recently Weinstock *et al.* (11) have announced that particulate matter of liver homogenates from tocopherol-deprived rabbits respired at increased rates when incubated under suitable conditions.

In the present investigation a significant alteration in the respiration of skeletal muscle and liver tissues from vitamin E-deficient rabbits was confirmed. The new observation that the oxygen consumption of the adrenal glands is also elevated will be presented. On the contrary, neither heart muscle nor kidney tissue appeared to undergo a significant variation in respiration. It will be indicated that calcium occupied a necessary rôle in the respiration increment of skeletal muscle, although calcium exerted an intense inhibitory effect on oxygen uptake. A similar depression of the oxygen consumption of liver and heart tissue by calcium was observed, but the latter had little influence on the respiration of kidney and adrenal preparations.

Methods

Young, white New Zealand rabbits (600 to 1200 gm.) were raised on the purified diet employed by Young and Dinning (12), which contains 15 per cent protein and the necessary quantities of starch, glucose, lard, cod liver oil, minerals, and crystalline vitamins to provide adequate but slow growth. The lard was assayed colorimetrically for tocopherols and

* This investigation was supported by a grant from the Muscular Dystrophy Associations of America, Inc.

[1] A preliminary report of this work was presented before the 126th meeting of the American Chemical Society, New York, September, 1954 (p. 58C, Abstracts of this meeting).

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was found to contain no detectable quantity of ferric chloride-dipyridyl-reacting substances. Withdrawal of tocopherol or cod liver oil afforded diets deficient in vitamin E and vitamin A + D, respectively. The addition of crystalline vitamin A to the diet lacking cod liver oil created a vitamin D-deficient diet.

The animals were kept in glass metabolism cages and the daily urine collected for creatine and creatinine determinations. The diacetyl method for creatine as used by Umbreit and Tonházy (13) for tissue studies was modified for the analysis of urinary creatine. A suitable urine aliquot, which was diluted 1:100 by the cage washings and additional distilled water, was allowed to react with 2 ml. of the alkaline naphthol and 1 ml. of the diacetyl reagent. The final volume was 6 ml., and after 10 to 15 minutes the 520 mμ absorption was measured in a Klett-Summerson photoelectric colorimeter. Creatinine estimations were performed via the Jaffe reaction.

The daily consumption of food and increase in body weight were recorded. Control animals were supplemented with 5 mg. of *dl*-α-tocopherol per day *per os*. Biopsies of skeletal muscle were taken at the time of sacrifice for histological estimation of the extent of nutritional muscular dystrophy. Samples of the rectus femoris from both legs were studied in the respirometry experiments.

Litter mate vitamin-deprived and vitamin-supplemented rabbits were sacrificed by stunning after 7 to 27 days on the diet. The tissues were sliced in a Stadie microtome, and muscle strips were prepared by gentle dissection with forceps. Adrenals were studied as bisected glands and as tissue preparations of separated adrenal cortex and adrenal medulla. Suitable quantities of tissue were incubated in a Krebs-Ringer-phosphate medium in the presence and absence of calcium at pH 7.32. Endogenous substrate of skeletal muscle was found to be low, and therefore incubations of all tissues were carried out with 0.1 ml. of 10 per cent glucose, which was tipped in from the side arm of 20 or 5 ml. vessels after a 15 minute equilibration period. Linear oxygen uptake was obtained in the first 20 minutes, and, although oxygen consumption was followed at least for 1 hour, Q_{O_2} was calculated from manometer changes observed at 4 minute intervals during the first 20 minutes. All determinations were performed in duplicate or triplicate.

Results

Since no description of the sequence of appearance of the pathological symptoms resulting from this purified diet has been published, a few descriptive findings will be mentioned. Similar to the observations on the classical vitamin E-deficient diets (14), the onset of overt symptoms (loss

of appetite, decline in body weight, and muscular paralysis) occurred in approximately 3 weeks. However, unlike the results with partially purified diets, a sharper end-point in the course of the disease was experienced. Within 2 to 3 days after a serious creatinuria was manifested (above 50 mg. per 24 hours), body weight declined, food consumption halted, and in many instances death ensued before muscular paralysis became evident. In the present study it was desired to determine oxygen consumption at a time when presumably few irreversible biochemical or physiological alterations might have occurred. Therefore the animals were sacrificed when urinary creatine levels were below 20 mg. per 24 hours. No signs of histological changes in skeletal muscle could be found under these conditions.

Accurate creatine estimations were obtained with the diacetyl method, but some comment of this procedure should be expressed. Erroneously high levels of urinary creatine were observed in three situations: (*a*) when urine was not sufficiently diluted, other amino acids may significantly react with the reagents (15); (*b*) when unusually high creatinine concentration exists, the creatine formed from the alkaline hydrolysis of creatinine will increase the color intensity; and (*c*) in animals with diarrhea there was profound increment in urinary material reacting with the diacetyl reagents. Animals exhibiting conditions (*b*) or (*c*) were rejected. Obviously, as creatinuria became elevated, further urinary dilution could be made. The diacetyl method has good sensitivity and was applied in the range of 10 to 20 γ. Approximately 80 rabbits have been studied, and the urinary creatine before the onset of nutritional muscular dystrophy ranged from 3 to 5 mg. per 24 hours. Creatinuria was clearly demonstrated by this method as the disease progressed. Recoveries of creatine from rabbit urine averaged 99 $\pm$ 6 per cent. The alkaline picrate procedure for creatinine indicated that little fluctuation of this urinary constituent (approximately 35 mg. per 24 hours) occurred during the course of the disease.

The earlier investigators of oxygen consumption in vitamin E-deprived animals utilized Locke's, Tyrode's, or Ringer's solution for their incubation media, and normally these all contain calcium. Although remarkable increments in Q_{O_2} on skeletal muscle were observed, absolute estimations of oxygen consumption appeared low for this tissue. At pH 7.32, the level used in the present study, calcium formed a precipitate, and it was decided to eliminate it from the Krebs-Ringer-phosphate buffer. It soon became obvious that little difference existed between the oxygen uptake of tocopherol-deficient and control skeletal muscle preparations (Table I) in the absence of calcium.

The effect of calcium ions on Q_{O_2} of skeletal muscle in phosphate buffer (turbid solution) and in Tris buffer (tris(hydroxymethyl)aminomethane, clear solution) is shown in Fig. 1. A serious depression in muscle respira-

tion for both deficient and control tissue can be seen. The decrement in oxygen uptake occurred in slices and strips and was independent of pH in the range 6.5 to 7.5. Serial dilutions of calcium concentration from 0.0027 to 0.0006 M resulted in a proportional descent in inhibition. Despite the inhibitor influence of calcium on Q_{O_2}, this mineral had to be included in the incubation medium in order to observe the increase in oxygen consumption of skeletal muscle from tocopherol-deficient rabbits (Table I).

By utilizing the most favorable conditions obtainable, the oxygen consumption of skeletal muscle, cardiac muscle, adrenal glands, kidney, and

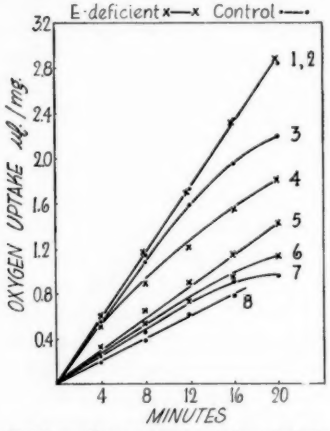

FIG. 1. The effect of Ca^{++} on the oxygen uptake of skeletal muscle in phosphate and Tris media. ×, vitamin E-deficient; ●, control. Curves 1 and 2, phosphate buffer without Ca^{++}; Curves 5 and 8, phosphate buffer with Ca^{++}; Curves 3 and 4, Tris buffer without Ca^{++}; Curves 6 and 7, Tris buffer with Ca^{++}.

liver from tocopherol-deprived and tocopherol-supplemented animals was compared. The results (Table I) reveal significant increases in the oxygen consumption of skeletal muscle, adrenal tissue, and liver slices derived from the vitamin E-deficient animals. Neither kidney nor heart slices appeared to vary appreciably from control levels. The oxygen consumption increment of adrenal and liver tissue remained apparent in the presence of calcium.

In regard to the high oxygen consumption levels in the adrenals, the question arises whether the alteration in adrenal respiration is directly related to tocopherol deprivation or to a stress complication or to both. Furthermore it was of interest to determine whether the increased oxygen uptake occurred in the adrenal cortex or medulla or both these endocrine tissues. A comparison of oxygen consumption levels of litter mate to-

copherol-deprived and tocopherol-supplemented rabbits is presented in Fig. 2. The oxygen consumption of adrenals from tocopherol-deprived animals exceeds that of the controls by 50 to 130 per cent and the difference between deficient and control adrenals occurs approximately at 14 days.

TABLE I

Oxygen Consumption of Tissues from Vitamin E-Deficient Rabbits

Tissue	Treatment	No. of rabbits*	Q_{O_2}, μl. per hr. per mg. dry weight			
			Calcium	Per cent change	No calcium	Per cent change
Skeletal muscle slices	Control	4	1.67 ± 0.48 (0.99–2.39)	+48	4.17 ± 0.56 (3.05–5.28)	±0
" " "	Deficient	5	2.48 ± 0.49 (1.76–3.31)		4.19 ± 0.59 (2.87–5.69)	
" " strips	Control	9	2.26 ± 0.74 (0.95–3.02)	+54	6.62 ± 1.17 (4.18–8.86)	+10
" " "	Deficient	12	3.48 ± 0.85 (2.42–5.78)		7.28 ± 1.30 (3.36–8.35)	
Adrenals, bisected	Control	13	2.53 ± 0.25† (2.27–2.77)	+71	2.44 ± 0.55 (1.26–3.92)	+104
" "	Deficient	9	4.35 ± 0.56† (3.78–4.91)		4.98 ± 1.22 (3.13–7.68)	
Kidney slices	Control	4	7.28 ± 0.82 (6.35–8.27)	−8	8.91 ± 2.19 (4.75–13.58)	−9
" "	Deficient	6	6.68 ± 1.31 (4.55–8.67)		8.04 ± 1.56 (5.71–12.68)	
Liver slices	Control	3	3.05 ± 0.77 (1.94–4.16)	+65	5.86 ± 1.63 (3.72–7.99)	+91
" "	Deficient	3	5.03 ± 1.04 (3.98–6.07)		11.23 ± 1.21 (10.46–12.01)	
Heart slices	Control	2	2.38 ± 0.21 (2.17–2.59)	+6	4.58 ± 0.57 (4.01–5.15)	±0
" "	Deficient	2	2.53 ± 0.25 (2.28–2.78)		4.60 ± 0.56 (4.04–5.16)	

* Tissue preparations from each rabbit were incubated either in duplicate or triplicate.

† These values are derived from only two animals.

The change in adrenal Q_{O_2} appeared before the variation in oxygen consumption of skeletal muscle was detected. Both histological and urinary creatine determinations indicated that no significant muscular alterations had ensued at the early emergence of the adrenal respiration increment.

The increased respiratory metabolism of the adrenal gland was found to occur in the adrenal cortex, as can be seen in Table II. The respiration

rates of adrenal cortical tissue from these animals and the controls on the purified diet were in the range of medullary preparations. Those of the

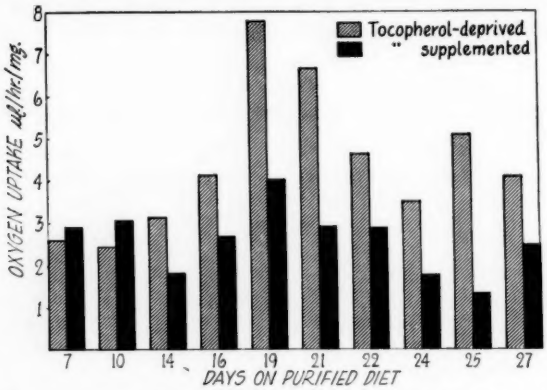

FIG. 2. A comparison of the oxygen consumption of adrenal tissue from tocopherol-deprived and tocopherol-supplemented rabbits.

TABLE II

Oxygen Consumption of Rabbit Adrenal Tissue

Treatment of rabbit litter mates	Q_{O_2}, μl. per hr. per mg. dry weight		
	Bisected glands	Adrenal cortex	Adrenal medulla
Normal stock diet	1.46 (1)*	2.03 ± 0.32 (8) (1.42–3.09)	1.71 ± 0.23 (8) (0.96–1.77)
Purified diet + tocopherol	2.44 ± 0.55 (13) (1.26–3.92)	1.30 (1)	1.19 (1)
Vitamin E-deficient	4.98 ± 1.22 (9) (3.13–7.68)	5.25 ± 1.10 (6) (3.06–7.85)	2.95 ± 1.56 (6) (0.96–5.00)
" A + D-deficient		10.27 ± 2.53 (3) (7.59–14.06)	1.30 ± 0.62 (3) (0.37–1.82)
" D-deficient		1.76 (1)	1.37 (1)
Pregnant, stock diet		4.99 ± 1.27 (2) (3.71–6.26)	1.70 ± 0.52 (2) (1.18–2.22)
Rabbit with broken leg, purified diet + tocopherol		7.74 (1)	0.86 (1)

* The figure in parentheses indicates the number of animals studied.

adrenal cortices from tocopherol-deficient rabbits were significantly elevated. A slight increment in the oxygen consumption of adrenal medulla from vitamin E-deficient animals was obtained, but may be due to incomplete separation of cortical and medullary tissue.

To determine whether vitamin E was responsible for maintaining normal oxidative processes in the adrenal or whether a non-specific chronic stress was exerted on the animals during a vitamin deficiency, rabbits deprived of vitamin A + D were studied under identical conditions. Table II clearly shows that vitamin A + D deficiency resulted in a remarkable increase in adrenal cortical respiration. A significant stress was not apparent in vitamin D-deprived animals after 21 days, probably because potential stores of vitamin D were available.

The stress of vitamin deficiency was also compared to that of pregnancy (rabbits on stock diet) and a control rabbit whose leg was inadvertently broken (Table II). The oxygen uptake of adrenal cortex in both these conditions was markedly increased.

DISCUSSION

A definitive explanation for the increased oxygen consumption of skeletal muscle is still to be welcomed. The rôle of calcium must be elucidated, but it is known that the activity of adenosinetriphosphatase is influenced by calcium (16). Calcium has also been implicated in other enzyme systems of muscle (17).

Although the earlier workers (1–9) employed calcium in their media, the oxygen uptake of their skeletal muscle preparations was determined between 2 and 3 hours after incubation commenced. It would seem that such estimations of oxygen consumption would sum all oxidative mechanisms, including the variant ones. In the present study it was found that linear oxygen uptake occurred in the first 20 minutes of incubation. Therefore measurements during this interval probably represent the more active oxygen-requiring reactions, which presumably are more closely related to the effects of tocopherol deficiency.

The mechanisms influenced in liver tissue in vitamin E deficiency remain to be illuminated. An increased synthesis or turnover of liver creatine has been suggested by the work of Roche *et al.* (18) who have reported a significantly higher incorporation of isotopic nitrogen into creatine in a case of progressive muscular dystrophy. Another possibility stems from the necessity of increased glucose synthesis for transport to muscle which is already depleted of glycogen and requires additional substrate for the processes involved in the elevated respiration.

The findings on the adrenal indicate that other vitamin deficiencies should have a similar effect on adrenal oxygen consumption which could in part be referred to a non-specific chronic stress. McKee and Walker (19) observed an elevated respiration in the adrenal cortices of scorbutic guinea pigs. Although additional definitive data are lacking in the literature, investigators have noticed that adrenal function is altered during the

deprivation of other water-soluble vitamins. Thus Olson and Deane (20) reported histological changes in the adrenals of choline-deficient weanling rats, and Forker and Morgan (21) found evidence of an impediment of the pituitary-adrenal cycle in riboflavin-deprived rats. It is conceivable that influences on adrenal oxygen uptake could be recorded before the occurrence of irreversible histological adrenal alterations in pantothenic deficiency (22).

A preferable time for the investigation of biochemical reactions during nutritional deficiencies would be before the appearance of marked symptoms of the disease, with the usual accompaniment of irreversible changes. The increase in adrenal Q_{O_2} as seen in the present study may be an appropriate index to such a suitable interval of study. Certainly in vitamin E deficiency the alteration in the adrenal respiration was observed in advance of all the previously observed pathological changes.

It was of interest to note the activity *in vitro* of corticotropin (ACTH) (2.5 mg. of LA-1-A[2] per pair of adrenals) on the adrenals of tocopherol-deficient rabbits. In two experiments ACTH increased the cortical respiration of vitamin E-deficient animals by 44 per cent, while the oxygen consumption of cortical slices of litter mate controls was raised by 85 per cent. Apparently there was an ability on the part of the adrenals to respond with a further increase in the already elevated oxygen uptake due to the vitamin deficiency. McKee and Walker (19) could not elicit a similar response with ACTH in their scorbutic guinea pigs. However, Quaife and Harris (23) obtained comparable adrenal ascorbic acid levels in vitamin E-deficient and control rats before and after adrenaline and cold stresses. This would agree with our finding that a reserve activity was available in the adrenals of the tocopherol-deprived animals.

The skilful technical assistance of Mr. Judson Spaulding is gratefully acknowledged.

SUMMARY

1. Significant increments in the Q_{O_2} of skeletal muscle, adrenal, and liver tissue of tocopherol-deprived rabbits were found. Little effect was observed for heart and kidney preparations.

2. Calcium was demonstrated to inhibit the Q_{O_2} of skeletal muscle, cardiac tissue, and liver slices, but, despite the inhibitory activity of calcium, the latter was necessary to reveal the increased Q_{O_2} of skeletal muscle.

3. An increase in oxygen uptake of the adrenals occurred in the adrenal cortex and was elicited by other vitamin deficiencies and stress situations.

[2] Corticotropin, Armour Laboratories.

4. The possible origin of the increase in respiration rates of tissues from tocopherol-deficient rabbits is discussed.

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PHOSPHOSERYLGLYCINE FROM DIISOPROPYLPHOSPHORYL CHYMOTRYPSIN AND INVERSION OF ITS PEPTIDE SEQUENCE BY ACID*

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Diisopropyl phosphorofluoridate (DFP) inactivates chymotrypsin to form the monophosphorylated derivative, diisopropylphosphoryl chymotrypsin (DPChtr). It has previously been reported (2) that, when this derivative was partially hydrolyzed with 2 N HCl at 100° for 18 hours, 30 per cent of the DFP-derived phosphorus could be accounted for as serine phosphoric acid. Most of the remaining phosphorus was identified as phosphoric acid. Two other organic phosphorus-containing fractions, each accounting for about 1 per cent of the total phosphorus, have now been identified as phosphoserylglycine and glycylserine phosphoric acid. The former has been shown to give rise to the latter under the conditions of the acid hydrolysis, and evidence is cited which indicates that only phosphoserylglycine represents the normal dipeptide sequence in DPChtr.

EXPERIMENTAL

P^{32}-Labeled DPChtr—This was prepared as described previously[1] (2), except that the major part of the unchanged P^{32} was removed by dialysis against distilled water rather than by salting out of the protein. The completeness of inactivation of chymotrypsin by DFP was checked by a determination of residual enzymatic activity (3); in all of our preparations this has amounted to a negligible part of the original activity.

Partial Hydrolysis of DPChtr and Dowex 50 Chromatography—DPChtr in a concentration of 1 to 1.5 per cent was hydrolyzed with 100 ml. of 2 N HCl at 100° for 2.5 hours. A precipitate which formed was removed by centrifugation and washed with water. The HCl was removed from the supernatant fluid and washings by repeated evaporation to dryness under reduced pressure. The residue was dissolved in 5 ml. of 0.05 N HCl[2] and chromatographed on a column of hydrogen form Dowex 50-X8 (200 to

* Presented in part at the Forty-fifth annual meeting of the American Society of Biological Chemists at Atlantic City (1).

[1] The following error was present in the description of the preparation P^{32}-labeled DPChtr: "34 ml. of P^{32}-labeled DFP" should read "34 mg. of P^{32}-labeled DFP."

[2] The solution contained a small amount of precipitate which was not separated.

XUM

400 mesh), 2.6 cm. in diameter and 100 cm. long, with 0.05 N HCl as the eluent at a flow rate varying from 18.5 to 61.5 ml. per hour in different preparations. Fractions, 2 or 4 ml. in volume, were collected with an automatic fraction collector. The phosphorus-containing substances thus separated were purified by rechromatographing them on a Dowex 50 column, 0.83 cm. in diameter and 100 cm. long, with 0.01 N HCl as the eluent at a flow rate of about 6 ml. per hour.

Paper Chromatography—Two-dimensional paper chromatograms were prepared with the phenol and 80 per cent pyridine solvents of Block *et al.* (4, 5) with 9 inch square chromatographic Whatman No. 1 filter paper and the capillary ascent method of Williams and Kirby (6). An amount of the phosphorus-containing substance equivalent to 1 γ of phosphorus was taken for chromatograms. Standard amino acids were used in 0.03 μmole quantities. The chromatograms were developed with the ninhydrin reagent of Block *et al.* (4). The position of the radioactivity was determined by directly monitoring the papers. The amino acid constituents of peptides were determined by chromatographing the sample after complete hydrolysis with 0.5 ml. of 6 N HCl at 100° for 16 to 20 hours.

Nitrogen Terminal Residues—Nitrogen terminal residues of peptides were determined with the fluoro-2,4-dinitrobenzene (FDNB) (7) and nitrosyl chloride (8, 9) methods.

In the FDNB method a sample of the peptide containing 4 γ of phosphorus was dissolved in 0.1 ml. of 1 per cent triethylamine, and to this was added a solution of 0.01 ml. of FDNB in 0.2 ml. of absolute ethanol. If the mixture was cloudy after shaking, a drop of ethanol was added. After standing overnight, 0.1 ml. of 1 per cent triethylamine was added, and the excess FDNB was extracted twice with 1 ml. of ethyl ether. The residue was evaporated to dryness and hydrolyzed with 0.5 ml. of 6 N HCl at 100° for 2.5 hours. When glycine was *N*-terminal, the hydrolysis time was reduced to 1 hour. After removal of the HCl one-half of the sample was chromatographed on paper.

In the nitrosyl chloride method a sample of the peptide containing 2 γ of phosphorus was dissolved in 0.03 ml. of 6 N HCl and transferred to the well of a microscope slide. The material was treated with nitrosyl chloride in a gassing apparatus for 15 minutes at room temperature, transferred to a 15 ml. centrifuge tube, and dried with a stream of air. The drying was repeated twice after the addition of 0.05 ml. of 6 N HCl each time. The resulting preparation was hydrolyzed with 0.5 ml. of 6 N HCl at 100° for 16 to 20 hours and chromatographed on paper.

Analytical Methods—The positions of P^{32}-containing fractions of the Dowex 50 chromatogram were determined by measuring the radioactivity of the solutions in test-tubes from the fraction collector when the tubes

were held in a rack in a fixed position against the window of the Geiger tube of a Tracerlab autoscaler. For accurate counting, aliquots of the fractions were made alkaline to phenolphthalein and evaporated to dryness in glass dishes of 1 inch diameter under an infra-red lamp.

Samples for microbiological assay were hydrolyzed with 0.5 ml. of 6 N HCl at 100° for 24 hours. Serine and glycine were determined with *Lactobacillus casei* and *Leuconostoc mesenteroides*, respectively. The serine values were corrected for an estimated 10 per cent destruction during the hydrolysis (10). Colorimetric phosphorus determinations were carried out by the method of Fiske and Subbarow (11). Nitrogen was determined by the semimicro-Kjeldahl method.

Results

Dowex 50 Chromatography of Partially Hydrolyzed DPChtr—The Dowex 50 chromatogram of the partial HCl hydrolysate of DPChtr showed five main phosphorus-containing fractions, only the last three of which are considered in this report. A representative chromatogram is presented in Fig. 1.

Nature of Phosphorus Fraction 3—Fraction 3 accounted for 27 to 34 per cent of the chymotrypsin-bound phosphorus in different preparations. A sample of this fraction, purified by rechromatographing twice as described above, contained 50.0 γ of nitrogen and 10.0 γ of phosphorus, giving a molar N:P ratio of 2.26. A paper chromatogram of this material showed a single ninhydrin spot just above the origin in the phenol direction, and the radioactivity coincided with the spot. The chromatogram of a completely hydrolyzed sample showed the presence of glycine and serine,[3] while the radioactivity remained at the origin (in the position of phosphoric acid). Paper chromatograms of the hydrolyzed dinitrophenyl derivative and the product deaminated with nitrosyl chloride showed glycine and a trace of serine.

Serine and glycine were determined on Fraction 3 by microbiological assay before and after deamination with nitrosyl chloride (Table I). The results indicate that these amino acids are present in equimolar ratio to each other and to phosphorus and that serine is lost after deamination.

As will be shown below, further hydrolysis of Fraction 3 with 2 N HCl yielded *O*-serine phosphoric acid as a degradation product. Only indirect evidence exists that the isopropyl groups originally present on the phosphorus of DPChtr have been removed by the hydrolysis. Diisopropylphosphoric acid yields about 80 per cent of its phosphorus as phosphoric

[3] All chromatograms of hydrolyzed fractions of serine phosphoric acid or its peptides show a small faint spot to the left and below serine (2). It is not ethanolamine phosphate and has so far not been identified.

acid when it is refluxed in 2 N HCl for 2.5 hours.[4] Phosphoric acid and serine phosphoric acid show no movement on paper with the solvents used here, whereas diisopropylphosphoric acid and its peptides exhibit considerable movement in each direction.[5] In this respect, Fraction 3 behaves more like a phosphate than a diisopropylphosphate ester.[6]

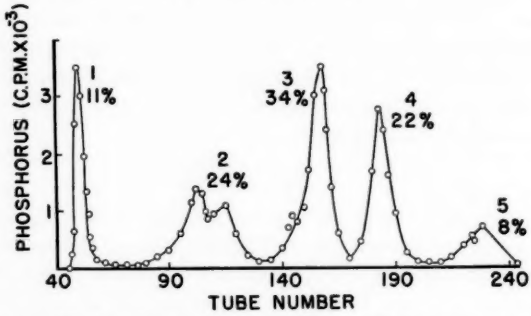

FIG. 1. Dowex 50 chromatogram of partial 2 N HCl hydrolysate of diisopropylphosphoryl chymotrypsin. 1.05 gm. of DPChtr hydrolyzed in 100 ml. of 2 N HCl at 100° for 2.5 hours. 99 per cent of phosphorus recovered. 200 to 500 mesh resin in hydrogen form. Column 2.6 × 100 cm. Eluent 0.05 N HCl. Flow rate 61.5 ml. per hour. Fraction size, 4 ml.

TABLE I

Amino Acid Composition of Phosphorus Fraction 3 by Microbiological Assay

	Amino acid	Amino acid found	Phosphorus content	Amino acid / Phosphorus
		γ	γ	*mole per mole*
Before deamination	Serine	16.1	5.6	1.2
	Glycine	9.3	4.1	0.9
After " *	Serine	1.3	3.1	0.1
	Glycine	9.0	3.1	1.2

* With nitrosyl chloride.

The above evidence indicates that Fraction 3 is a dipeptide of serine phosphoric acid and glycine and that the former is *N*-terminal; hence it is phosphoserylglycine.

[4] Data of Dr. R. E. Plapinger of these laboratories.

[5] Unpublished experiments.

[6] We have obtained an unidentified phosphorus fraction in small yield from the 2.5 hour 2 N HCl hydrolysate of DPChtr, the direct chromatogram of which shows considerable movement with the present solvents.

Nature of Phosphorus Fraction 4—This fraction, obtained in a 22 to 33 per cent yield, would be expected to be serine phosphoric acid, since its position on the Dowex chromatogram corresponds to that of serine phosphoric acid previously identified after prolonged acid hydrolysis of DPChtr (2). A paper chromatogram of this fraction showed a single ninhydrin spot near the origin, coinciding with the radioactivity. The completely hydrolyzed fraction showed only serine, and this was no longer present on the chromatograms of the hydrolyzed dinitrophenyl derivative and deaminated fraction.

Nature of Phosphorus Fraction 5—This fraction was obtained in a 7 to 8 per cent yield. A paper chromatogram showed a single ninhydrin spot just above the origin, and the radioactivity coincided with the spot. On complete hydrolysis it showed glycine and serine, but the hydrolyzed dinitrophenyl derivative and deaminated fraction showed serine with only a trace of glycine.

TABLE II

Amino Acid Composition of Phosphorus Fraction 5 by Microbiological Assay

	Amino acid	Amino acid found	Phosphorus content	Amino acid / Phosphorus
		γ	γ	*mole per mole*
Before deamination	Serine	9.9	4.4	0.7
	Glycine	9.5	4.4	1.1
After " *	Serine	10.2	4.4	0.7
	Glycine	2.3	4.4	0.2

* With nitrosyl chloride.

The serine and glycine contents of this fraction by microbiological assay before and after deamination are reported in Table II. The results indicate that these amino acids are present in an equimolar ratio to each other and to phosphorus and that glycine is lost after deamination. Since the amino group of glycine is free, the phosphorus can only be in ester linkage with the hydroxyl group of serine. Hence this fraction is glycylserine phosphoric acid.

Partial Conversion of Phosphoserylglycine to Glycylserine Phosphoric Acid by 2 N HCl Hydrolysis—The possibility existed that either the phosphoserylglycine or the glycylserine phosphoric acid arose from the other substance by a diketopiperazine rearrangement, such as has been described recently by Sanger and Thompson for glycylvaline (12). To test this possibility a preparation of P^{32}-labeled phosphoserylglycine, *i.e.* Fraction 3, which gave a single peak on Dowex 50 chromatography and contained 356 γ of phosphorus, was hydrolyzed in 42.5 ml. of 2 N HCl at 100° for 2.5 hours. The

HCl was removed, and the residue was chromatographed on Dowex 50-X8 with 0.01 N HCl as the eluent. Four P^{32} fractions were obtained (Fig. 2). The probable structure of these fractions derived from evidence of paper chromatography is presented in Table III. Fraction 2 was indistinguishable from synthetic serine phosphoric acid (13) in an isotope dilution experiment in which both were chromatographed together on Dowex 50 with 0.01 N HCl as the eluent (2). It should be noted, incidentally, that the eluent, 0.01 N HCl, reverses the order of elution of serine phosphoric acid and phosphoserylglycine as compared with the eluent, 0.05 N HCl, which was used with the original hydrolysate.

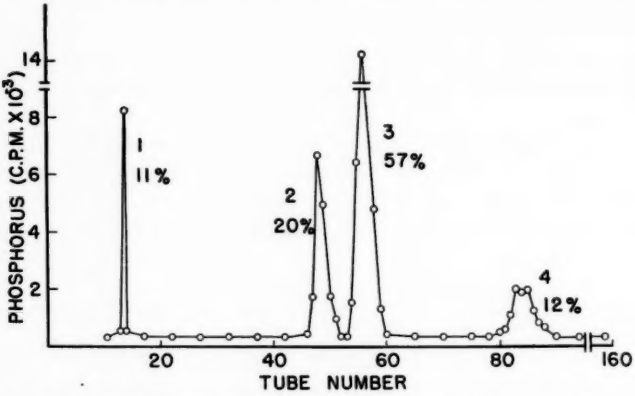

FIG. 2. Dowex 50 chromatogram of partial 2 N HCl hydrolysate of phosphoserylglycine. See Table III for the nature of the fractions. 200 to 400 mesh resin in hydrogen form. Column 0.83 × 100 cm. Eluent 0.01 N HCl. Flow rate 6 ml. per hour. Fraction size, 2 ml.

TABLE III

Phosphorus Fractions of Partial HCl Hydrolysate of P^{32}-Labeled Phosphoserylglycine from Evidence of Paper Chromatography

Fraction No.	Ninhydrin spots before hydrolysis*	Amino acids after complete hydrolysis	Amino acids after hydrolysis of dinitrophenyl derivative	Probable structure
1	None	None		Phosphoric acid
2	1 at origin	Serine	None	Serine phosphoric acid
3	1 " "	" glycine	Glycine	Phosphoserylglycine
4	1 " "	" "	Serine	Glycylserine phosphoric acid

* The position of the radioactivity was at the origin with each fraction before and after hydrolysis.

The above experiment, indicating the formation of glycylserine phosphoric acid during the partial HCl hydrolysis of phosphoserylglycine, was repeated twice with similar results. The ratio of the yields of phosphoserylglycine and glycylserine phosphoric acid from these three experiments was 3.8, 3.9, and 4.6, or an average of 4.1. The corresponding ratios of the yields from the first Dowex 50 chromatogram of two DPChtr hydrolyses were 4.0 and 4.8. These results are consistent with the postulation of an acid rearrangement of these dipeptides.

DISCUSSION

In view of the demonstration of the partial conversion of phosphoserylglycine to glycylserine phosphoric acid under the conditions used for the hydrolysis of DPChtr, either one of these dipeptides in the DPChtr hydrolysate may arise from the other secondarily through a diketopiperazine rearrangement. Diketopiperazine formation has not been found with concentrated acid and low temperature (14). In work to be published later, we have hydrolyzed DPChtr with 12 N HCl at 37° for 3 days and have found phosphoserylglycine and other phosphopeptides, but no glycylserine phosphoric acid. Under these conditions it was shown that phosphoserylglycine did not yield glycylserine phosphoric acid, which is in agreement with the similar finding of Sanger and Thompson with glycylvaline (12). Therefore, phosphoserylglycine represents the normal dipeptide sequence in DPChtr and glycylserine phosphoric acid is an artifact.

It is obvious that caution must be exercised in deducing normal peptide sequences from dipeptides isolated from protein hydrolysates when these hydrolysates are prepared at 100° with 2 N or more dilute HCl. For example, this might well raise a question concerning the true amino acid sequence in casein on the basis of the isolation of the phosphopeptide phosphoserylglutamic acid by 2 N HCl hydrolysis from this protein (15).

SUMMARY

Diisopropylphosphoryl chymotrypsin was partially hydrolyzed with 2 N HCl at 100° for 2.5 hours. Serine phosphoric acid, phosphoserylglycine, and glycylserine phosphoric acid were separated from the hydrolysate by Dowex 50 chromatography. Phosphoserylglycine, hydrolyzed under the same conditions, was partly converted to glycylserine phosphoric acid. Evidence is cited that only phosphoserylglycine represents the normal dipeptide sequence in diisopropylphosphoryl chymotrypsin.

The authors are indebted to Dr. B. J. Jandorf for guidance and advice, to Dr. H. O. Michel for the determination of DFP purity and chymotrypsin

activity, to Dr. R. E. Plapinger for the synthetic serine phosphoric acid, and to Mrs. Dorothy V. Johnson for the microbiological assays.

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THE USE OF BIOLOGICALLY PREPARED RADIOACTIVE INDICATORS IN METABOLIC STUDIES: METABOLISM OF PENTOBARBITAL*

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Although isotopic labeling has been of great use in studies of drug metabolism, the necessarily small amounts of material used in such experiments often preclude the identification of unknown metabolites by conventional chemical means. Such difficulties are commonly circumvented by adding a known non-labeled compound, suspected to be a metabolite, to the unknown mixture and reisolating the substance in labeled form. The isotope dilution technique, however, involves guesses about the unknown structures which may not be correct, especially when metabolic alteration has obscured the relationship between precursor and metabolite. There are a number of recorded instances in which difficulties in predicting structure have hampered or prevented the identification of unknown derivatives (2, 3).

The use of biologically prepared radioactive indicators to guide the isolation of sufficient material for direct chemical identification offers one means of avoiding structural assumptions. The indicators are prepared as radiochemically pure entities by microfractionation (usually paper chromatography) of the labeled metabolites obtained after administering a radioactive precursor of high specific activity. A single indicator may be added to urine or other large pools of degradation products from animals receiving the unlabeled parent compound, and its radioactivity used to guide fractionation. As in the conventional isotope dilution procedure, isolation of radioactive material satisfying the criteria of homogeneity demonstrates the derivation of the product from the parent substance.

When the desired products are present in low concentration, very large pools of material may have to be used to provide tangible quantities in the final isolation. By means of counter-current distribution (4), separations carried out on a small aliquot of the pooled metabolites may be exactly duplicated with any desired volume. Parallel distributions of a small indicator-labeled aliquot and the main body of material may thus be run

* A preliminary report was presented before the Federation of American Societies for Experimental Biology, Chicago, April 6–10, 1953 (1).

until the preparation has been sufficiently freed of extraneous material to permit combination of the two aliquots.

Although this approach to metabolic problems represents a relatively obvious application of the principles of the isotope dilution procedure, it does not appear to have been generally realized that preliminary microfractionation of labeled metabolic indicators and use of the latter in conjunction with counter-current distribution can greatly facilitate the application of the isotope dilution principle to substances of unpredictable structure occurring in small amounts.

The interest of this laboratory in barbiturate metabolism prompted a study of the fate of pentobarbital (Nembutal) as a demonstration of the applicability of this procedure.

When urine from dogs receiving pentobarbital-2-C^{14} was examined by paper chromatography, a total of ten radioactive substances was revealed. Of these, one was recovered pentobarbital and three could be accounted for by derivatives previously identified. Two diastereoisomeric forms of 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid have been isolated from dog urine by Maynert and Dawson (5), and one of these has also been identified by Mark *et al.* (6) as a metabolite of pentobarbital in man. Maynert and van Dyke (3), on the basis of studies with N^{15}-labeled pentobarbital, have concluded that a small amount of the drug is converted to urea.

Other paper chromatographic experiments by Roth *et al.* (7) have indicated the existence of five metabolites of pentobarbital-2-C^{14} in rat urine. Algeri and McBay (8) have offered chromatographic evidence for one acidic metabolite in ether extracts of dog and human urine in addition to the diastereoisomeric 3-hydroxy derivatives.

This report is concerned with the isolation and characterization of 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid, the first of the unknown metabolites of pentobarbital to be identified by this procedure. An analogous acid has been identified as a metabolite of thiopental in man by Brodie *et al.* (9).

Evidence has also been obtained that a second highly water-soluble metabolite is a glucuronide of the dextrorotatory form of 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid. It would appear that the discrepancy in excretion of the *d*- and *l*-hydroxy derivatives, which account for about 21 and 41 per cent, respectively, of the metabolites of pentobarbital, may result from preferential conversion of the former to a glucuronide.

EXPERIMENTAL

Preparation of Pentobarbital-2-C^{14}—In a tube protected from moisture a mixture of 497 mg. of diethyl ethyl 1-methylbutyl malonate, 60 mg. (1 mc.) of urea-C^{14}, and 1.5 ml. of 2.5 M sodium ethoxide in anhydrous ethanol was

evaporated almost to dryness under a stream of dry nitrogen. After the tube was sealed, heated at 80° for 2 hours, and cooled, its contents were dissolved in 2 ml. of cold 1 N NaOH and extracted several times with ether to remove unchanged ester. Of the 146 mg. (63 per cent yield) of pentobarbital in the alkaline solution (estimated by ultraviolet absorption at 255 mμ) approximately 114 mg. precipitated as an oil when 0.35 ml. of glacial acetic acid was added. After recrystallization from water, this melted at 129–130°. Homogeneity of the radioactive material was established by both paper chromatography and a twenty-four transfer Craig counter-current distribution (4). For the latter a sample of 0.37 mg. (1.7 μc.) of radioactive pentobarbital was mixed with 10 mg. of unlabeled carrier to avoid erratic results due to the adsorption of minute amounts of pentobarbital on the steel surfaces of the Craig apparatus. Distribution was carried out between $CHCl_3$ and buffer of pH 9.5 prepared by mixing 10 volumes of a solution 1 M in both boric acid and KCl with 6 volumes of 1 N NaOH.

Determination of C^{14}—Samples were plated directly in cup planchets and counted with an end window counter having a background of approximately 20 c.p.m. All counts were corrected for self-absorption. For direct counting of urine a correction curve for various weights of urinary solid was obtained by evaporating samples of normal dog urine with a standard number of counts. Since it was difficult to avoid uneven deposition of solids from evaporating urine, this procedure was not entirely satisfactory. Individual determinations, however, were reproducible within approximately 10 per cent.

For direct counting from paper chromatograms, 1 cm. squares were cut out and counted individually in a gas flow proportional counter (10) with a background of approximately 2 c.p.m.

Paper Chromatography—Samples were chromatographed on Whatman No. 1 or No. 3 paper with *n*-butanol saturated with 1 per cent ammonia as the descending solvent (7).

Similar systems have been used by Algeri and McBay (11) for the identification of barbiturates in medicolegal problems. Radioautographs were prepared by exposure on Eastman type K industrial x-ray film.

Occasional chromatograms of non-radioactive material were sprayed with a 0.1 saturated solution of cobaltous acetate in pyridine. Pentobarbital and its derivatives gave a dull purple color with this reagent.

Recovery of Administered Radioactivity—Fig. 1 represents the recovery of radioactivity in urine after approximately 5 μc. of pentobarbital-2-C^{14}, diluted with sufficient unlabeled drug to provide a dose of 30 mg. per kilo, were administered intravenously to a dog. Within the uncertainty imposed by the self-absorption corrections, recovery was essentially complete within 15 to 20 hours.

In a subsequent experiment designed to provide large amounts of labeled metabolites, a similar dose of sodium pentobarbital containing 0.37 mc. of activity was administered intravenously to a dog, and urine was collected for 5 days. In this experiment the dog was maintained in an air-tight cage for 24 hours, and expired CO_2 was collected in an absorption train containing 2.9 liters of 2 N NaOH. Conversion to $BaCO_3$ for counting yielded 233 mg. per ml. of alkaline solution. Radioactivity was barely detectable in the precipitate; a sample of about 30 mg. registered 2 c.p.m. over a background of 1.8 in the Robinson counter (10). It was estimated that the expired CO_2 could not have accounted for more than 0.008 to 0.009 mc. (0.21 to 0.24 per cent) of the administered activity.

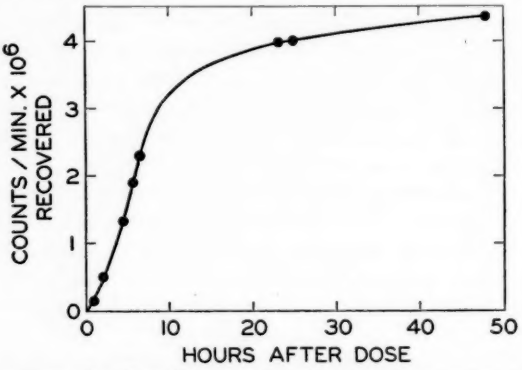

FIG. 1. Recovery of radioactivity in dog urine after intravenous administration of 4×10^6 c.p.m. of pentobarbital-2-C^{14}. Aliquots of 0.5 ml. of urine were evaporated for direct counting. Counts corrected for self-absorption.

Fractionation of Radioactive Metabolites—The radioautogram obtained when 0.1 ml. of the urine from the 0.37 mc. experiment was chromatographed on Whatman No. 1 paper is reproduced in Fig. 2. A total of ten spots was observed. For the sake of convenience in referring to individual fractions, these were numbered in order of decreasing R_F. In order to ascertain the relative proportions of the metabolites, 1 cm. squares of the paper were cut out for individual counting. The results together with the identities of the derivatives are recorded in Table I. For the isolation of usable amounts of radiochemically pure fractions, the urine at pH 7 was extracted six times with an equal volume of *n*-butanol, the extract evaporated *in vacuo*, and the residue subjected to a ten transfer counter-current distribution in 25 ml. layers of mutually saturated *n*-butanol and 0.25 M pyrophosphate buffer, pH 9.4. This sufficed to separate the bulk of the radioactive material in tubes 3 to 7 from much of the extraneous urinary

solids. The contents of tubes 3 to 7 were concentrated in this as well as in subsequent distributions by acidifying the aqueous layer, extracting with butanol several times, and evaporating the solvent *in vacuo* at 40°. Material thus obtained was subjected to a 124 transfer distribution in the

FIG. 2. Radioautogram from a chromatogram on Whatman No. 1 paper of 0.03 ml. of urine from a dog receiving 0.37 mc. of pentobarbital-2-C^{14}. Developing solvent, *n*-butanol saturated with 1 per cent NH_4OH. Component 1 is the unaltered pentobarbital. The major metabolites (Components 2 and 3) are the *d* and *l* forms of 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid. Component 7 is urea. Component 8 is identified in this study as 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid, and Component 10 is shown to be a glucuronide of Component 2. The remaining substances are unidentified.

same solvents by the single withdrawal procedure (12) in a Craig all-glass apparatus (Fig. 3). Paper chromatography of selected fractions from this run indicated partial separation of the metabolites. Tubes 101 to 124 contained only Component 1; tubes 58 and 62 contained mixtures of Components 2 and 3; tube 34, a mixture of Components 3, 4, and 5; tube 22, Component 5 with small amounts of Components 6, 7, and 8; and tube 16, a mixture of Components 5 through 8. By combining material in the vicinity of the above tubes, the contents of the Craig apparatus were di-

vided into fractions for further resolution by chromatography on large sheets of Whatman No. 3 paper. Streaks representing the individual

TABLE I

Recovery of Metabolites after Chromatography of 0.1 Ml. of Urine from Dog Receiving Pentobarbital-2-C^{14} on Whatman No. 1 Paper (Fig. 1)

Developing solvent, *n*-butanol saturated with 1 per cent NH_4OH.

Spot No.	R_F	C.p.m. in spot	Per cent of recovered activity	Identity
1	0.86	144	0.8	Pentobarbital
2	0.63	3685	21.0	*d* alcohol*
3	0.54	7209	41.0	*l* "
4	0.46	268	1.5	Unknown
5	0.26	182	1.0	"
6	0.18	374	2.1	"
7	0.12	464	2.6	Urea
8	0.07	790	4.5	Carboxylic acid†
9	0.03	2195	12.4	Unknown
10	0.01	2405	13.6	Glucuronide of Spot 2

* 5-Ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid.

† 5-Ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid.

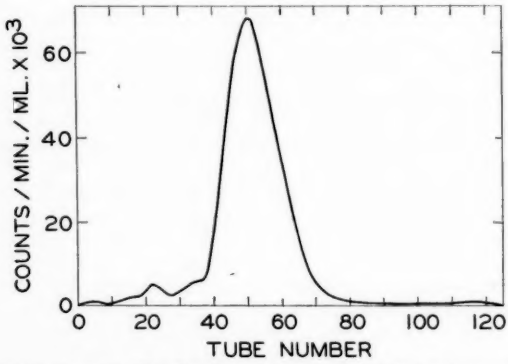

FIG. 3. Counter-current distribution (124 transfers) in *n*-butanol and 0.25 M pyrophosphate, pH 9.4, of urinary metabolites of pentobarbital-2-C^{14}. Tubes 101 to 124 contained unchanged pentobarbital; tubes 58 and 62, mixtures of Components 2 and 3; tube 34, a mixture of Components 3, 4, and 5; tube 22, mostly Component 5 plus some Components 6, 7, and 8; tube 16, a mixture of Components 5 through 8.

radioactive components were located by radioautography and eluted with 85 per cent methanol.

In order to resolve the more water-soluble derivatives remaining in the

urine after the butanol extraction and in tubes 8 to 10 of the first counter-current distribution, this material was combined and concentrated, methanol was added to precipitate salts, and the residue, after evaporation of the mother liquor, was fractionated by large scale paper chromatography.

With the exception of the apparently very labile Component 9, which did not survive the repeated refractionations, the labeled metabolites were obtained as radiochemically pure entities.

Identification of Metabolites—The identities of several components, Nos. 1, 2, 3, and 7, were readily apparent from preliminary experiments. Component 1 (R_F 0.87) proved indistinguishable from pentobarbital, both chromatographically and by counter-current distribution in the $CHCl_3$ buffer system.

The relative abundance (Table I) of the major metabolites, Components 2 and 3, suggested their identity with the *d* and *l* forms, respectively, of 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid. These substances were reported by Maynert and Dawson (5) to represent 15 and 33 per cent of the administered pentobarbital. They were isolated from the urine of dogs receiving 7 gm. of unlabeled pentobarbital by a process of extraction and counter-current distribution essentially the same as that described above for the radioactive urine. Material from tubes 3 to 7 of a ten transfer distribution in 100 ml. volumes of the two solvents was redistributed in 25 ml. volumes. The contents of tubes 2 to 9 of the second run were then finally distributed in the Craig 100 plate glass apparatus. By fractional crystallization of material from the large central fraction in 50 per cent ethanol, samples of the *d* and *l* alcohols melting at 208.5–211° and 152.5–153.5°, respectively (reported, 209–210° and 152–153°) (5), were obtained. These were chromatographically indistinguishable from radioactive Components 2 and 3, respectively, and showed the characteristic ultraviolet absorption spectrum ($\lambda_{max.}$ 255 mμ in alkali) and the strong hydroxyl band at 2.8 μ in the infra-red described by Maynert and Dawson for these compounds.

The R_F value of 0.12 for Component 7 was identical with that of urea, and a mixture of the radioactive metabolite with urea-C^{14} gave a single radioactive spot when chromatographed on paper. From the urine of the first experiment, in which the dog received 3×10^6 c.p.m. of radioactive pentobarbital, Component 7 was obtained by a series of butanol extractions and chromatography of the evaporated extracts on several sheets of Whatman No. 3 paper. Urea, m.p. 134°, obtained by methanol elution of the appropriate area of paper, was recrystallized from ethanol to a constant specific activity of 190 c.p.m. per mg. in the low background (2 c.p.m.) counter. The xanthydrol derivative of this was prepared (13) in glacial acetic acid, washed with ethanol, and recrystallized twice from pyridine to a specific activity of 31 c.p.m. per mg. (calculated 27 c.p.m.).

Attention was next turned to the components which remained to be identified. Preliminary work with radioactive samples indicated that, although Component 8 could be quantitatively extracted from solutions of pH less than 3 into ether, the more polar alcoholic solvents were required to remove appreciable quantities of Components 4 through 6. None of the extremely water-soluble Component 10 was extractable by either type of solvent.

Efforts to isolate Components 4 and 5 from butanol extracts of urine have not yet been successful, largely because of the difficulties in separating them from the greatly preponderant alcohol derivatives. Enough of the more abundant Component 8, however, has been obtained from this source to permit characterization, and its isolation is described.

In order to provide material for larger scale isolation experiments, the pooled urine from a group of dogs which had received a total of approximately 50 gm. of unlabeled sodium pentobarbital was evaporated *in vacuo* to 4650 ml. The concentrate was adjusted to pH 3.0 with 150 ml. of HCl, and a 48 ml. (0.01) aliquot was removed for pilot experiments to be described below.

A sample of 26,600 c.p.m. of radioactive Component 8 was added to the 48 ml. aliquot. This was extracted three times with equal volumes of *n*-butanol and twice with isobutanol.

After evaporation of the butanol extracts *in vacuo* at room temperature, the residue was dissolved in a minimum of water, acidified with HCl, and extracted several times with an equal volume of ether. After these extracts were evaporated and the solid residue triturated several times with ether, 15,500 c.p.m. (58 per cent) were recovered in the final ether solution.

In order to guide further fractionation, this aliquot containing 15,500 c.p.m. was evaporated and subjected to a twelve transfer counter-current distribution in 4 ml. layers of *n*-butanol and 0.5 M borate buffer, pH 8.2. Samples of 0.5 ml. were taken from each layer for counting (Fig. 4). By selecting the more water-soluble fractions in tubes 11 and 12, it was possible to obtain approximately 6700 c.p.m. in a fraction essentially free from the more abundant alcohol derivatives concentrated in tubes 3 to 10. The material thus obtained was then distributed with eight transfers and 4 ml. each of the two phases obtained by mixing equal volumes of water, methanol, chloroform and ether (Fig. 4). This concentrated 71 per cent of the remaining radioactivity into tubes 2 to 6 and permitted a considerable amount of colored contaminants to be discarded with the end tubes. Tubes 2 to 6 now contained 18 per cent of the radioactivity originally present.

These pilot operations were then exactly duplicated on a 100-fold scale with the main body of urine. An oil, estimated on the basis of the accompanying radioactive experiments to contain about 18 per cent of the metab-

olite originally present in urine, was obtained by concentration of tubes 2 through 6 of the final distribution.

A 24 mg. aliquot of this oil was labeled with approximately 40,000 c.p.m. of Component 8 and carried through a twenty-four transfer distribution in the Craig apparatus with the four solvent system. Individual tubes were assayed by evaporation of suitable aliquots for counting, and the residues were dissolved in 1 N NaOH for measurement of ultraviolet absorption. Although the radioactivity distributed as a component with a partition

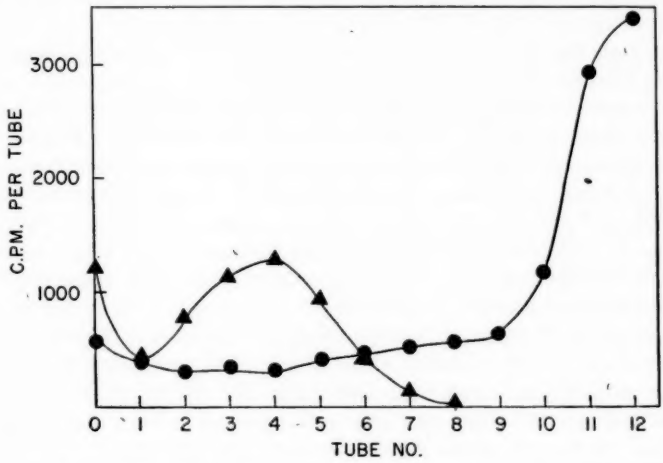

FIG. 4. Counter-current distribution of an aliquot of butanol extract from urine of dogs receiving pentobarbital. ●, aliquot labeled with radioactive Component 8 distributed in butanol-0.5 M borate, pH 8.2. ▲, tubes 11 and 12 redistributed in two phases obtained by mixing equal parts of $CHCl_3$, methanol, H_2O, and ether.

coefficient (K) of 1 (maximal concentration in tube 12), the ultraviolet-absorbing material exhibited a broad band corresponding to $K = 0.7$ (maximal concentration in tube 10). Maximal absorption of the alkaline solution occurred at 280 mμ.

On the basis of this pilot distribution, the main portion of oil was subjected to a 200 transfer counter-current distribution in the mixture of four solvents used before. Individual tubes were assayed by evaporation of suitable aliquots, solution of the residues in 1 N NaOH, and measurement of the ultraviolet absorption at 255 and 280 mμ. As expected from the preliminary run, a fraction exhibiting maximal concentration at tube 100 and distributing in good agreement with the partition coefficient of 1 for Component 8 was partly separated from approximately 480 mg. of a

contaminant which had its maximal concentration in tube 79 and which distributed in accordance with a partition coefficient of 0.65. The amount of contaminant was estimated by inspection of the distribution curve according to conventional procedures (Weissberger (12) p. 194). The value for the extinction coefficient ($E^{1\%}_{1\,cm.}$ = 1550 in 1 N NaOH at 280 mμ) used in these calculations was obtained from a sample of the contaminant isolated by evaporation of appropriate fractions and recrystallized from water to a constant melting point of 202–203.5°. In acid $\lambda_{max.}$ for this substance was 255 mμ. It is not a metabolite of pentobarbital and was not further characterized.

Although the fractions containing Component 8 absorbed strongly at 255 mμ in alkali, as expected for a barbiturate, there was spectral evidence of impurities absorbing at shorter wave-lengths. Tubes 79 to 100, which would contain one-half of a substance with a distribution coefficient of 1, were evaporated. One-tenth of the residue, to which had been added 4970 c.p.m. of Component 8, was dissolved in a little 0.1 N sodium formate buffer, pH 4.08, and placed on an 0.8 cm. × 2 cm. column of Dowex 1-X2 (2 per cent cross-linked resin) prepared as the formate (14). After traces of material having ultraviolet absorption properties similar to those of pentobarbital had been washed from the column with 60 ml. of the buffer, elution with 20 ml. of 0.1 N formic acid removed a small band of an unidentified contaminant. Further elution with 40 ml. of the acid then removed a sharply defined band containing 4000 c.p.m. and 30 mg. of an acidic substance with an absorption spectrum indistinguishable from that of pentobarbital; *i.e.*, $\lambda_{max.}$ in alkali 255 mμ; no absorption in acidic solution. On the basis of these experiments it was estimated that approximately 600 mg. of Component 8 had been present in the original oil.

This metabolite was more easily obtained in crystalline form from the contents of tubes 100 to 118 of the 200 transfer counter-current distribution. These were evaporated to dryness and taken up in a little acetone. Addition of several volumes of ether precipitated some brown amorphous material, which was discarded. After evaporation of the filtrate *in vacuo* the residue was taken into a little hot 95 per cent ethanol and allowed to stand in the cold for 7 days. Four recrystallizations of the precipitate from 65 per cent ethanol yielded crystalline material melting at 194–196° (Kofler hot stage). Precipitation was slow, 3 to 4 days being required for each recrystallization. The ultraviolet absorption spectrum in 1 N NaOH could not be distinguished from that of pentobarbital. Titration of a 12 mg. sample with standard alkali gave a curve with points of inflection at pH 6.4 and 9.8. The pK_a of the stronger acid function was calculated to be 4.5, characteristic of a carboxylic acid. Both the molecular weight of 263 calculated from the titration data and the analytical results were in

agreement with the formulation of the product as 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid.

257.3. Calculated, C 51.55, H 6.29; found, C 51.39, H 6.33

This structure was confirmed by comparison with synthetic material, kindly prepared by Dr. Harry B. Wood and Dr. E. C. Horning (15). Synthetic acid melting at 192–194° did not depress the melting point of the isolated metabolite. The infra-red absorption spectra and x-ray powder diagrams of the two compounds were identical. The synthetic acid, the isolated metabolite, and the radioactive fraction designated as Component 8 all showed partition coefficients of 1 in the two-phase system prepared by mixing methanol, chloroform, ether, and water (1:1:1:1) and 0.8 between ether and buffer at pH 4.56 prepared by mixing 46 volumes of 0.2 M Na_2HPO_4 and 54 volumes of 0.1 M citric acid.

Since increased glucuronide excretion has been reported to follow administration of pentobarbital to dogs (5), it was presumed that the extremely water-soluble Component 10 might be such a conjugate. This was confirmed by incubating approximately 25,000 c.p.m. of the chromatographically pure radioactive metabolite with 100 mg. of bacterial β-glucuronidase (Sigma) in 0.1 M phosphate buffer at pH 6.15. After 14 hours at 37°, protein and salt were precipitated by the addition of several volumes of methanol. The precipitate was washed with a little methanol, and the combined solution and washings were evaporated *in vacuo*. The residue was separated into two fractions by a twelve transfer counter-current distribution between 5 ml. layers of a system prepared by mixing 2 volumes of acetone with 10 volumes each of *n*-butanol and 0.1 M phosphate buffer, pH 7.48. The hydrolysis product occurring as a fraction in tubes 0 to 4 (Fig. 5) amounted to 14 per cent of the total counts. The distribution data would permit this to be Component 2, 3, or 4 but would exclude Component 5. When isolated and chromatographed on paper, this material migrated as a single spot indistinguishable from Component 2, the *d* alcohol of Maynert and Dawson. Since the presence of impurities extracted from the enzyme incubation mixture caused slight smearing of the paper chromatogram, it was not possible to exclude the possible presence of some of the *l*-hydroxy derivative (Component 3) in the hydrolysate.

Because of the low yield of free alcohol obtained by enzymatic hydrolysis, it was considered desirable to repeat the incubation with glucuronidase. The unchanged material in tubes 6 to 12 was therefore evaporated *in vacuo*, taken up in a little water, adjusted to pH 6, and treated with enzyme as before. A second distribution gave results essentially the same as the first, 13.5 per cent of the counts appearing as the free alcohol in tubes 0 to 4.

It would thus appear that under the conditions employed here the

enzymatic hydrolysis of the glucuronide is either very slow or comes to an equilibrium favoring the conjugated form.

These experiments suggested that preferential conversion of the *d* alcohol to a glucuronide might explain the greater recovery of the *l* compound

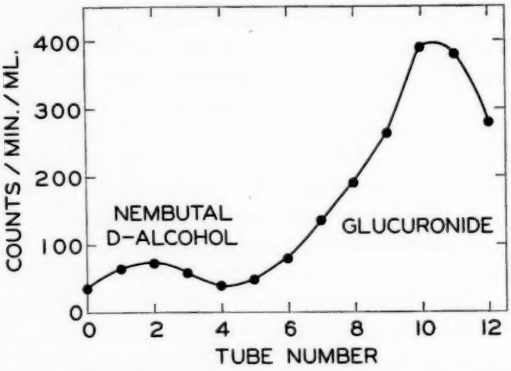

FIG. 5. Counter-current distribution of reaction products from Component 10 and glucuronidase in two-phase system obtained by mixing butanol, acetone, and phosphate buffer (pH 7.48) (10:2:10).

TABLE II

Recovery of Radioactivity in Dog Urine after Administration of d- and l-5-Ethyl-5-(3-hydroxy-1-methylbutyl)barbituric Acid-2-C^{14}

d, 1.08 × 10^6 c.p.m.		*l*, 4.0 × 10^6 c.p.m.	
Hrs. after injection	C.p.m. ± 10 per cent	Hrs. after injection	C.p.m. ± 10 per cent
0– 2	3.0 × 10^5	0– 3	1.79 × 10^6
2– 4	2.13 × 10^5	3–19	8.75 × 10^5
4– 6	1.64 × 10^4	19–46	2.12 × 10^5
6–48	1.80 × 10^3	46–84	7.25 × 10^3
48–52	875		
Total.....	710,275		2,884,250

noted by Maynert and Dawson and confirmed by the observations of the present authors. In order to test this, 1.08 × 10^6 c.p.m. of chromatographically pure *d*-5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid-2-C^{14} were diluted to 8 mg. with unlabeled compound and injected intravenously into a dog. Urine was collected by catheterization for the first few hours and subsequently from the cage. 2 weeks later 4.0 × 10^6 c.p.m. of the corresponding *l* alcohol were also diluted to 8 mg. with carrier and similarly

injected. Samples of 0.5 ml. of urine were taken for counting until activity fell to about twice the background. The weights of residual urinary solid for which correction had to be made varied from 20 to 82 mg., a range in which the uncertainty in the corrected counts was consistently about 10 per cent. Approximately 71 per cent of the administered activity was recovered in each experiment (Table II).

Samples of the two urines were acidified with HCl and extracted five times with equal volumes of ether in order to remove the unchanged alcohols from the water-soluble conjugates. Of 289,500 c.p.m. in an aliquot of urine from the experiment with the *d* alcohol, 69,500 c.p.m. (24 per cent) remained in the aqueous phase. Of 1.96×10^6 c.p.m. in an aliquot from the other urine, 104,000 c.p.m. (5.3 per cent) were conjugated.

Samples of the water-soluble fractions were prepared for paper chromatography by lyophilization and trituration of the solid residues with methanol. The radioactive spots, like Component 10, did not move appreciably in the butanol-ammonia system.

Except for one unidentified fraction with an R_F of approximately 0.1, the activity in the ether extracts was chromatographically indistinguishable from that of the alcohols originally administered. The unidentified fraction never exceeded 3 per cent of the ether-extractable activity.

DISCUSSION

When lack of appropriate model compounds prevents the identification by isotope dilution of unknown metabolites derived from an isotopically labeled precursor, recourse must be had to direct isolation of identifiable quantities of the labeled derivatives. This may be impractical with the more potent pharmacological agents, which can be administered only in small amounts or when the metabolite under investigation represents a small proportion of the administered precursor. In some instances, larger doses or repeated doses of the substance to be studied can provide larger amounts of metabolite, but dilution of the isotope limits the extent to which these expedients may be carried and excessive doses frequently cause a change in the pattern of metabolites produced, as is the case with epinephrine (16). The latter situation is particularly to be avoided in pharmacological studies, in which knowledge of the metabolic fate of a drug may be important to an understanding of its mode of action. It is in the hope of providing a means for identifying metabolites of substances which are physiologically effective in small amounts that the procedure involving preliminary fractionation of biologically prepared indicators has been suggested.

The present work on the metabolism of pentobarbital was undertaken to demonstrate the applicability of the procedure.

By means of the method a metabolite analogous in structure to the carboxylic acid derivative of thiopental described by Brodie *et al.* (9) has been

isolated. This type of oxidation is consistent with the fact that many compounds carrying alkyl groups are metabolized by terminal oxidation of the side chain (17).

The isolation of 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid probably accounts for the ether-extractable acidic metabolite of R_F 0.08 for which Algeri and McBay (8) have recently offered paper chromatographic evidence. The absorption maximum at 256 mμ in acid reported by the above authors probably reflects the presence of the unidentified contaminant melting at 203.5°. The latter exhibited remarkable similarity in many properties to the acid metabolite, and in our hands the two compounds were separable only by resorting to a 200 transfer distribution.

SUMMARY

The use of biologically prepared radioactive indicators in a modification of the isotope dilution procedure, designed to facilitate the identification of unknown metabolites occurring in small amounts, has been described.

Paper chromatography of urine from dogs receiving pentobarbital-2-C^{14} revealed nine metabolites. With the aid of the indicator procedure, one of these has been isolated and identified as 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid.

Of the two previously known diastereoisomers of the 3-hydroxy derivative of pentobarbital, the dextrorotatory form appears to be preferentially conjugated with glucuronic acid.

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STUDIES ON THE BIOSYNTHESIS OF SERINE*

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Formaldehyde has emerged as the primary precursor of the β-carbon of serine (1–4), although in certain systems the β-carbon can be formed from formate (5–7), methanol (2), and the methyl groups of methionine (8), sarcosine (3), and acetone (9). It is also evident that the activated 1-carbon moiety is not formaldehyde *per se*, but some derivative thereof. A clue to the nature of the active 1-carbon unit was furnished by experiments on the effect of folic acid deficiency on serine formation in bacteria (10, 11) and in the rat (12) and the chick (13).

It had been suggested earlier by Gordon *et al.* (14) that folic acid may serve as the cofactor for 1-carbon compound reactions in purine and histidine biosynthesis. It seemed reasonable, therefore, to assume a similar rôle for folic acid (or its derivatives) in serine biosynthesis. This has received support from the recent reports of Kisliuk and Sakami (15, 16) and Blakley (17) indicating that THFA[1] is the carrier coenzyme of formaldehyde in serine biosynthesis.

In addition to this, Berg (18, 19) has shown a specific effect of homocysteine on formate incorporation into serine *in vitro*. Deodhar and Sakami (20) also observed this same effect with formaldehyde.

Another of the B vitamins, pyridoxal, has been implicated in the biosynthesis of serine. Lascelles and Woods (21) first observed its requirement for serine synthesis by *Streptococcus faecalis*, and Deodhar and Sakami (20) reported that pyridoxine-deficient chicks have a diminished ability to incorporate formate-C^{14} into serine.

The purpose of the present work was to investigate the mechanism of serine synthesis from formaldehyde and glycine in a soluble rat liver preparation. Preliminary reports of the work have been presented (22, 23).

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[1] THFA = tetrahydrofolic acid; Tris = tris(hydroxymethyl) aminomethane; ATP = adenosine triphosphate; DPN, DPNH = diphosphopyridine nucleotide and the reduced form.

EXPERIMENTAL

Enzyme Preparation—The soluble enzyme extract is the same as the crude enzyme preparation of Cantoni (24) and was prepared from the livers of unfasted, adult, female rats (200 to 250 gm.) of the Long-Evans strain, sacrificed by cervical fracture. The livers of the only other two animal species tested, guinea pig and sheep, contained the enzyme.

A lyophilized preparation was obtained by dialyzing the clear extract for 15 to 20 hours against four changes (13 volumes each) of 0.01 M Tris buffer at pH 7.2 in the cold. The dialyzed solution was then lyophilized and stored in dry form at −15° (160 gm. of liver yield 9 gm. of dry powder). Even at this temperature, the material on aging loses activity which can be restored with reduced glutathione (see "Results"). The preparation was weighed and dissolved in Tris buffer just prior to use.

Method of Assay—Most of the incubations were carried out in 20 ml. beakers at 37° in a Dubnoff metabolic incubator (25). To maintain the anaerobic conditions (26) essential when THFA was made, a stream of nitrogen was continuously passed through a dry box. These particular incubations were performed in tightly stoppered 25 ml. Erlenmeyer flasks filled with N_2 gas and shaken mechanically in a constant temperature (37°) water bath.

At the end of the incubation time, the reaction was stopped by addition of trichloroacetic acid (final concentration 6 per cent), and 100 to 125 mg. of carrier serine were added. Subsequently, the serine was recrystallized from alcohol-water to constant specific activity, as previously described (at least five recrystallizations) (3, 7). It had been shown, and confirmed by us at the beginning, that when formaldehyde-C^{14} was the isotopic substrate the radioactivity was all present in the β-carbon of the serine (2, 3), and therefore periodate degradation (27) was unnecessary. Usually, about 30 mg. of serine were plated on round, tared aluminum planchets (16.0 sq. cm. by 0.2 cm.) in the form of a suspension in 1.0 ml. of 95 per cent ethanol. The samples were allowed to dry by evaporation (1 to 2 hours), often with the aid of an infra-red lamp (15 to 20 minutes drying time) until constant weight was obtained.

The radioactivity was measured with an end window counter and scaler of the Nuclear Instrument and Chemical Corporation or a Tracerlab gas flow counter and scaler. Corrections for self-absorption were applied in the calculations. The activities are expressed as recovered counts and per cent recovery (Equations 1 and 2).

(1) $$\text{Recovered counts} = \text{specific activity} \times \text{amount of added carrier}$$

(2) $$\%\ \text{recovery} = \frac{\text{recovered counts} \times 100}{\text{total counts added}}$$

Results

Experiments with Crude, Non-Dialyzed Extracts

Anaerobically, the non-dialyzed enzyme preparation converts formaldehyde to the serine β-carbon in significant amounts (Table I). Aerobically, the conversion is not nearly as good, which is probably due to the instability in air of the cofactor THFA derived from endogenous citrovorum factor (*cf.* Jukes (26)). The methyl group of methionine is utilized to a very limited extent and only aerobically. This is reasonably explained on the

TABLE I

Conversion of Various Substrates to β-Carbon of Serine

Each flask contained 1.5 ml. of non-dialyzed enzyme preparation, 10 μmoles of glycine, 1.0 ml. of 0.1 M glycylglycine buffer, pH 7.4. Total volume, 3.0 ml. Incubated for 2 hours at 37°. 10 μmoles of each substrate were used. $H_2C^{14}O$ = 189,000 c.p.m.; methionine-$C^{14}H_3$ = 1,650,000 c.p.m.; $H_3C^{14}OH$ = 330,000 c.p.m.; $HC^{14}OOH$ = 6,000,000 c.p.m.

Experiment No.	Substrate	Atmosphere	Recovered counts	Per cent recovery
1	$HC^{14}HO$*	N_2	0	0
2	"	"	18,000	9.6
3	"	Air	5,700	3.0
4	Methionine-$C^{14}H_3$*	"	85	
5	"	"	6,000	0.4
6	"	N_2	0	
7	$HC^{14}OOH$	Air	0	0
8	"	N_2	3,020	0.5
9	$H_3C^{14}OH$	Air	0	0
10	"	N_2	770	0.2

* No enzyme added.

basis of the need for oxygen for the oxidation of the methyl group to formaldehyde. Similarly, traces of formate and methanol are utilized anaerobically. Glycine-2-C^{14}, betaine-$C^{14}H_3$, sarcosine-$C^{14}H_3$, and choline-$C^{14}H_3$ are not utilized at all, either under anaerobiosis or aerobiosis.

The reverse reaction, serine $\rightleftharpoons$ glycine + formaldehyde, was catalyzed only to a limited extent (see Table II). The discrepancy of the results observed in using β- and carboxyl-labeled serine could be due to a loss of formaldehyde through volatilization.

Since our preparation was identical with the one used for methylating nicotinamide, it was of interest to learn whether any of the compounds required in that system would have any effect. As seen in Table III, there is a marked similarity in the two systems. ATP, Mg^{++}, and methionine

activate the crude enzyme system of this reaction. Activation by ATP had previously been observed with formaldehyde by Mitoma and Greenberg (3) with mitochondrial preparations. A similar activating effect with formate has also been reported (15–17). Homocysteine can replace methionine, and its activating effect has been demonstrated before (18–20) by other workers. Hence, it is believed that the action of methionine is actually mediated after its conversion to homocysteine, in which ATP and Mg^{++} are required (24). But, as will be seen later, the effect of ATP can also be due to its rôle in the formation of THFA from endogenous citrovorum factor. It is interesting that DL-ethionine inhibits the activation by methionine and that L-methionine counteracts it in this system (Table III, Experiments 6 and 7).

TABLE II

Formation of Glycine and Formaldehyde from Serine

Each flask contained 1.5 ml. of non-dialyzed enzyme preparation, 1.0 ml. of 0.1 M phosphate buffer, pH 7.4. Incubation under N_2 for 2 hours at 37°. Total volume, 3.0 ml. Serine-$C^{14}OOH$ = 40,000 c.p.m.; serine-3-C^{14} = 100,000 c.p.m.

Experiment No.	Contents	Recovered counts	Per cent recovery
1	10 μmoles DL-serine-$C^{14}OOH$	600*	1.5
2	10 " DL-serine-3-C^{14}	275†	0.3

* Recovered as glycine by separation on a Dowex 50 column (28).
† Recovered as methylenebismethone (3).

TABLE III

Effect of Activators on Serine Biosynthesis

Each flask contained 1.5 ml. of non-dialyzed enzyme preparation, 1.0 ml. of 0.1 M glycylglycine buffer, pH 7.4, 10 μmoles of $H_2C^{14}O$ (187,000 c.p.m.), 20 μmoles of Mg^{++}, 10 μmoles of ATP, 8 μmoles of DL-methionine unless indicated otherwise. Total volume, 3.0 ml. Incubated under N_2 for 2 hours.

Experiment No.	Contents	Recovered counts	Per cent recovery
1	Complete system	37,750	19.8
2	No Mg^{++}	20,000	10.0
3	" ATP	18,500	9.8
4	" methionine	21,000	11.0
5	" Mg^{++}, ATP, methionine	19,000	10.0
6	Complete + 25 μmoles DL-ethionine	18,000	9.6
7	" + 25 " " + 10 μmoles L-methionine	36,500	19.3

Figs. 1 and 2, *A* present graphically the effect of varying concentrations of the different activators. The inhibiting effect of DL-methionine at higher concentrations is due to the presence of the D isomer, as seen in the curve when L-methionine was used. DL-Homocysteine behaves in the same way that L-methionine does. The effects of increasing ATP, Mg^{++}, and glycine concentrations are all similar.

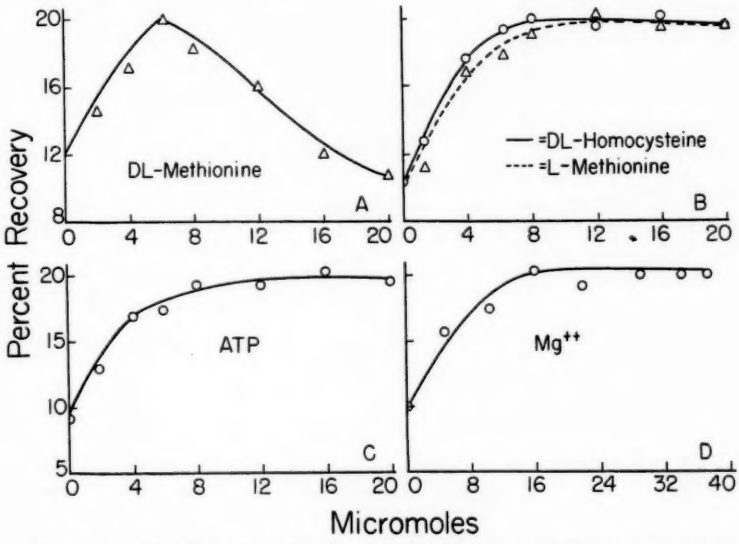


FIG. 1. Serine synthesis as a function of varying concentrations of DL-methionine (*A*), L-methionine and DL-homocysteine (*B*), ATP (*C*), and Mg^{++} (*D*). Each flask had 1.0 ml. of 0.1 M glycylglycine buffer, pH 7.4, 10 μmoles of $H_2C^{14}O$, 10 μmoles of L-methionine, 20 μmoles of Mg^{++}, 10 μmoles of ATP, 1.5 ml. of enzyme solution, except as indicated. Incubated under N_2 for 2 hours. Total volume, 3.0 ml. $H_2C^{14}O$ = 189,000 c.p.m.

The optimal pH-activity range was 7.0 to 7.4. Glycylglycine was found to be a better buffer medium than phosphate, Tris, bicarbonate, or maleate, because of the extra free glycine formed in it. As indicated by Fig. 2, *A* the optimal amount of serine synthesis occurs when at least 30 μmoles of glycine are present. When Tris buffer was used, 45 μmoles of glycine were employed.

A balance study of the reaction was carried out in the manner described by Mitoma and Greenberg (3) and is presented in Table IV. Anaerobically, 72.6 per cent of the total added radioactivity remained as unchanged formaldehyde and 21.05 per cent was found in serine. Aerobically, 77.7

per cent was recovered as formaldehyde and 3 per cent was found in serine. Some of the radioactivity unaccounted for could be attributed to loss by volatilization and fixation or incorporation into other undetermined compounds.

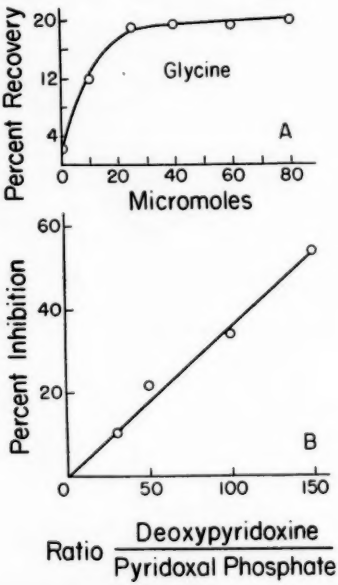


FIG. 2. *A*, serine biosynthesis as a function of glycine concentration. Conditions as in Fig. 1, except that 1.0 ml. of 0.1 M Tris buffer, pH 7.4, was used. *B*, inhibition ratios of deoxypyridoxine to pyridoxal phosphate. Preincubation was carried out with 25 μmoles of deoxypyridoxine and pyridoxal phosphate at the indicated ratios in the presence of 25 μmoles of ATP with crude enzyme extract (1.0 ml.). The complete incubation mixture was then added: 10 μmoles of $H_2C^{14}O$, 45 μmoles of glycine, 1.0 ml. of 0.1 M Tris buffer, pH 7.2. Incubated under N_2 for 2 hours. $H_2C^{14}O$ = 189,000 c.p.m.

Freezing and thawing had no effect on the activity of the enzyme preparation, making it amenable to lyophilization.

Experiments with Dialyzed-Lyophilized Enzyme Preparation

Reactivation by Leucovorin (Synthetic Citrovorum Factor) and ATP Together and by THFA Alone—Following dialysis and lyophilization, the activity of the preparation is almost nil. The activity is restored by ATP and leucovorin together and neither alone has any effect (Table V). Homocysteine and methionine have no effect either singly or in combination with

ATP and ATP + leucovorin. In confirmation of the results of Kisliuk and Sakami and of Blakley (15–17), we found that THFA alone is sufficient to reactivate the preparation. On the other hand, folic acid alone, or with ATP in the presence of reducing agents or reduced DPN cannot replace leucovorin, but boiled liver juice can serve as a partial replacement. At least 50 mg. of lyophilized enzyme preparation are required for optimal activity (Fig. 3, *C*).

Fig. 3, *B* is a plot of serine synthesis as a function of the leucovorin concentration. The plateau is reached at about 350 γ of leucovorin. Thereafter all of the experiments were conducted with 400 γ of leucovorin. The

TABLE IV

Balance Study of Conversion of Formaldehyde-C^{14}

Warburg flasks were used with KOH in the center well: 10 μmoles of $H_2C^{14}O$ (18,000 c.p.m.), 20 μmoles of Mg^{++}, 8 μmoles of ATP, 8 μmoles of DL-methionine, 10 μmoles of glycine, 1.0 ml. of 0.1 M glycylglycine buffer, pH 7.4, and 0.5 ml. of enzyme solution. Incubated under N_2 for 2 hours. Total volume, 3.0 ml.

Experiment No.	Compound	Nitrogen		Air	
		Recovered counts	Per cent recovery	Recovered counts	Per cent recovery
1	CO_2	27	0	270	0.15
2	Protein	228	1.25	141	0.8
3	Formaldehyde	13,100	72.6	14,000	77.7
4	Formate	0	0	0	0
5	Serine	3,800	21.05	520	3.0
Total		17,155	94.9	14,931	81.65

comparatively high leucovorin concentration requirement may be due to the mixture of diastereoisomers, only one of which is biologically active (30).

Fig. 3, *D* shows the effect of the time of incubation on serine formation. The experiment was carried out for 12 hours. The striking extent of formaldehyde conversion into serine, as much as 70 per cent conversion at the end of 12 hours, is demonstrated in this experiment. Bacterial action is not involved; if it were, the curve would exhibit an induction phase.

The effect of ATP is presumably concerned with the conversion of leucovorin (*N*-5-formyl-5,6,7,8-tetrahydrofolic acid) to THFA (5,6,7,8-tetrahydrofolic acid), the probable cofactor required for the activation of formaldehyde in the biosynthesis of serine. This conclusion, of course, is based on the observation that THFA does not require the presence of ATP, whereas leucovorin does.

Speculation on the rôle of ATP leads to the suggestions that ATP can

(1) act as an energy source for the conversion of leucovorin to THFA in the removal of the formyl group; (2) contribute a molecular species, either itself or a breakdown product, which can act as an acceptor for the formyl group of leucovorin. The latter supposition has the merit of some experi-

TABLE V

Effect on Serine Synthesis of Leucovorin, Folic Acid, and ATP

Each flask contained 45 μmoles of glycine, 10 μmoles of $H_2C^{14}O$ (31,000 c.p.m.), 1.0 ml. of 0.1 M Tris buffer, pH 7.2. Total volume, 3.0 ml. Incubated under N_2 for 3 hours. 50 mg. of lyophilized enzyme (4 days old) were dissolved in 1.0 ml. of Tris buffer (THFA prepared according to Broquist *et al.* (29)).

Experiment No.	Contents	Recovered counts	Per cent recovery
1	Control	630	1.9
2	" + 10 μmoles ATP	600	1.9
3	" + 400 γ leucovorin	630	1.9
4	" + 400 " " + 10 μmoles ATP	11,500	36.0
5	" + 400 " " + 8 " DL-homocysteine	540	1.7
6	Control + 400 γ leucovorin + 8 μmoles L-methionine	600	1.9
7	Control + 10 μmoles ATP + 8 μmoles DL-homocysteine	580	1.8
8	Control + 10 μmoles ATP + 8 μmoles L-methionine	720	2.3
9	" + 400 γ folic acid	660	2.1
10	" + 400 " " " + 10 μmoles ATP	670	2.1
11	" + 400 " " " + 10 " " + 8 μmoles DL-homocysteine	420	1.3
12	Control + 400 γ folic acid + 10 μmoles ATP + 8 μmoles L-methionine	600	1.9
13	Control + 400 γ folic acid + 1 mg. DPNH	660	2.1
14	" + 400 " " " + 1 " " + 10 μmoles ATP	660	2.1
15	Control + 10 μmoles ATP + boiled liver juice	5,950	19.1
16	" + 400 γ THFA	11,000	35.5
17	" + 400 " " + 10 μmoles ATP	11,400	36.7

mental support from the observation of Buchanan and Schulman (31) on the "enzymatic exchange" of formate with carbon atom 2 in inosinic acid that is specifically activated by leucovorin. It is conceivable that the system contains enzymes which split the purine ring to give "formate" plus 4-amino-5-imidazolecarboxamide-5′-phosphoribotide. Once this occurs, leucovorin can donate its formyl group to resynthesize inosinic acid and generate THFA. The THFA is then made available to act as the cofactor in the synthesis of serine, or (3) act by a combination of (1) and (2), since,

as has been pointed out in the work of G. R. Greenberg (32, 33), energy from ATP and the presence of leucovorin are required for purine synthesis from formate and the ribotide.

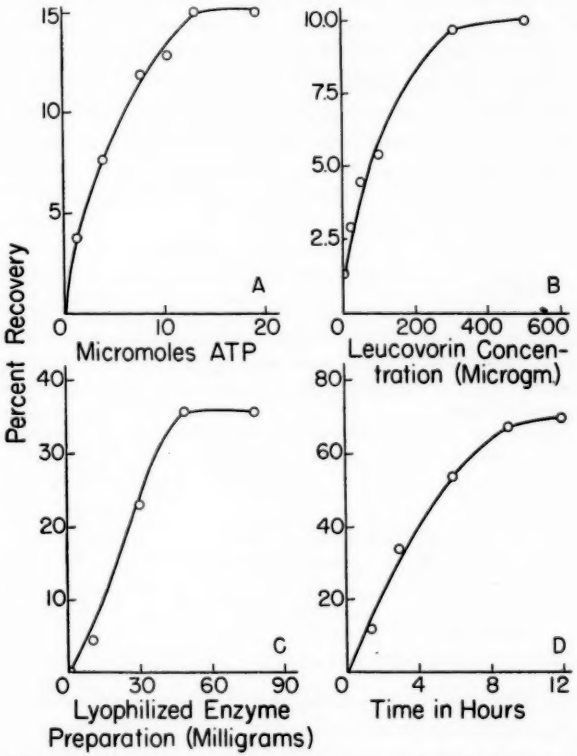


FIG. 3. Effect of various factors on serine biosynthesis with dialyzed, lyophilized enzyme preparation. Each experiment contained 1.0 ml. of 0.1 M Tris buffer, pH 7.2, 45 μmoles of glycine, 10 μmoles of $H_2C^{14}O$, 400 γ of leucovorin (except as indicated in *B*), 10 μmoles of ATP (except in *A*), 50 mg. of lyophilized enzyme (except in *C*) dissolved in 1.0 ml. of Tris buffer just prior to use. Total volume, 3.0 ml.; N_2 gas phase. All incubations were for 3 hours except for *B*, which lasted 1.5 hours, and *D* as indicated. $H_2C^{14}O$ = 32,000 c.p.m. except in the leucovorin experiments (*B*) in which the activity was 43,000 c.p.m.

Possibility (1) was tested by measuring the amount of serine synthesis as a function of the ATP concentration (Fig. 3, *A*). Inasmuch as serine synthesis is proportional to the amount of ATP present, it is safe to assume that ATP is required as an energy source in the conversion of leucovorin

to THFA. This energy is not needed for the transfer of the formaldehyde group to THFA (to form 5-hydroxymethyl-5,6,7,8-THFA) and thence to serine, because incubations with THFA require no ATP.

However, this does not exclude a rôle of either function (2) or (3), because, although the concentration of the molecular species furnished by ATP for the acceptance of the formyl group should not approach substrate amounts, energy may be required in the reaction involving its "formylation" by leucovorin. Contrary to the latter reasoning, when inosinic acid, adenylic acid, 5-amino-4-imidazolecarboxamide (the ribotide was not available), and guanylic acid were tried as substitutes for ATP, none of them was effective (Table VI). It might be more illuminating if the experiment

TABLE VI

Inability of Different Purine Nucleotides to Replace ATP

All flasks contained 50 mg. of lyophilized enzyme, 10 μmoles of $H_2C^{14}O$ (36,000 c.p.m.), 45 μmoles of glycine, 400 γ of leucovorin, 1.0 ml. of 0.1 M Tris buffer. Total volume, 3.0 ml. Incubated under N_2 for 1 hour. The enzyme (53 days old at −15°) was dissolved in 1.0 ml. of Tris buffer and activated with 5 μmoles of reduced glutathione just prior to use.

Experiment No.	Contents	Recovered counts	Per cent recovery
1	Control	3750	10.3
2	" + 10 μmoles inosinic acid	55	0.2
3	" + 10 " guanylic acid	24	0.1
4	" + 10 " adenylic "	106	0.3
5	" + 10 " 5-NH_2-4-imidazole-carboxamide	82	0.2

were repeated by using limiting amounts of ATP and another energy source; *e.g.*, phosphocreatine or phosphoglycerate with muscle extract.

Evidence for Involvement of —SH Enzyme—The enzyme preparation loses activity at −15°. This activity is restored by treatment with reduced glutathione, but not with vitamin B_{12} (Table VII). Free —SH groups are required for activity when either ATP and leucovorin or THFA alone is used as cofactor. Consequently, the need for free —SH groups cannot be attributed to the enzyme system converting leucovorin to THFA and must be assigned to the enzyme (or enzymes) responsible for the synthesis of serine from formaldehyde and glycine.

Confirmatory inhibition studies with hydrogen peroxide and *p*-chloromercuribenzoate are presented in Table VIII. Although hydrogen peroxide is not a specific oxidizing agent for the —SH group, the reversal of inhibition by reduced glutathione is indicative that —SH groups are being

oxidized. The more specific —SH reagent, *p*-chloromercuribenzoate, offers confirmatory information. Complete reversal of the inhibition by reduced glutathione, because of the competition with the inhibitor, is re-

TABLE VII

Reactivation of Aged (25 Days at −15°) Lyophilized Enzyme by Reducing Agents

Each flask contained 400 γ of leucovorin, 10 μmoles of ATP, 45 μmoles of glycine, 10 μmoles of $H_2C^{14}O$ (43,000 c.p.m.), 1.0 ml. of 0.1 M Tris buffer, pH 7.2, and 50 mg. of lyophilized enzyme (25 days old) dissolved in 1.0 ml. of Tris buffer just prior to use. Incubated under N_2 for 1.5 hours. Total volume, 3.0 ml.

Experiment No.	Contents	Recovered counts	Per cent recovered
1	No glutathione	2000	4.7
2	+ 5 μmoles reduced glutathione	5750	13.4
3	+ 5 " ascorbic acid	5200	12.1
4	+ 1 mg. vitamin B_{12}	2250	5.2
5*	No glutathione	2300	6.3
6*	+ 15 μmoles glutathione	8650	23.7

* Experiments 5 and 6 contained 400 γ of THFA, 45 μmoles of glycine, 10 μmoles of $H_2C^{14}O$ (36,500 c.p.m.), 1.0 ml. of 0.1 M Tris buffer, pH 7.2, and 50 mg. of lyophilized enzyme (90 days old) dissolved in 1.0 ml. of Tris buffer just prior to use. Incubated under N_2 for 3 hours. Total volume, 3.0 ml.

TABLE VIII

Inhibition of Serine Synthesis by —SH Reagents; Reversal by Glutathione

Each flask contained 45 μmoles of glycine, 10 μmoles of $H_2C^{14}O$ (36,500 c.p.m.), 400 γ of leucovorin, 10 μmoles of ATP, and 50 mg. of lyophilized enzyme (80 days old). The control (Experiment 1) was left at room temperature for 1 hour in 1.0 ml. of buffer prior to incubation. Total volume, 3.0 ml. including 1.0 ml. of 0.2 M Tris buffer, pH 7.2.

Experiment No.	Contents	Per cent recovery
1	25 μmoles glutathione	21.5
2	25 μeq. H_2O_2 (1 hr. at room temperature)	3.8
3	25 " " + 25 μmoles glutathione	18.2
4	1 μmole *p*-chloromercuribenzoate (1 hr. at room temperature)	10.2
5	1 μmole *p*-chloromercuribenzoate + 35 μmoles glutathione	22.2

alized only with a high concentration of reduced glutathione. With hydrogen peroxide, to restore full activity only enough glutathione is necessary to neutralize the amount of the oxidant because of the concomitant formation of the innocuous products: water and oxygen.

The need for —SH groups by this aldehyde-condensing enzyme is reminiscent of other —SH-requiring aldehyde enzymes (*cf.* Barron (34)).

TABLE IX

Inhibition of Serine Synthesis by Deoxypyridoxine (DPX) and Reversal by Pyridoxal Phosphate

In Experiments 2 to 6 the flasks contained 1 ml. of crude enzyme extract preincubated for 1 hour at 37° with DPX and 25 μmoles of ATP plus the indicated compounds. The control flask (Experiment 1) was preincubated with 25 μmoles of ATP only. After preincubation, the complete incubation mixture was added; 1 ml. of 0.1 M glycylglycine buffer, pH 7.4, 10 μmoles of $H_2C^{14}O$ (70,000 c.p.m.), and 10 μmoles of glycine. Incubated under N_2 for 1 hour. Total volume, 3.0 ml. In Experiments 7 to 12 the flasks contained 50 mg. of lyophilized preparation (90 days old) dissolved in 1.0 ml. of 0.2 M Tris buffer, pH 7.2, and preincubated for 1 hour at 37° with 25 μmoles of ATP and 25 μmoles of DPX plus pyridoxal phosphate (B_6-PO_4) when indicated. The controls (Experiments 7 and 10) were preincubated with 25 μmoles of ATP only. After preincubation, 1.0 ml. of 0.2 M Tris buffer, pH 7.2, 10 μmoles of $H_2C^{14}O$ (37,500 c.p.m.), and 45 μmoles of glycine were added. 400 γ of leucovorin and 10 μmoles of ATP were added in Experiments 7 to 9 and 400 γ of THFA in Experiments 10 to 12. 15 μmoles of reduced glutathione were used to reactivate the enzyme in all flasks. Incubated under N_2 for 2 hours.

Experiment No.	Contents	Recovered counts	Per cent inhibition
1	Control (crude extract)	4,950	0
2	" " " + 25 μmoles DPX	2,500	50
3	" " " + 25 " " + 5 μmoles pyridoxal	2,300	53.7
4	Control (crude extract) + 25 μmoles DPX + 5 μmoles pyridoxamine	555	88.9
5	Control (crude extract) + 25 μmoles DPX + 5 μmoles pyridoxine	1,300	73.7
6	Control (crude extract) + 25 μmoles DPX + 5 μmoles B_6-PO_4	4,700	5
7	Leucovorin control (dialyzed preparation)	3,400	0
8	" " " " + 25 μmoles DPX	1,260	63
9	Leucovorin control (dialyzed preparation) + 5 μmoles B_6-PO_4	3,430	0
10	THFA control (dialyzed preparation)	11,000	0
11	" " " " + 25 μmoles DPX	6,300	47
12	THFA control (dialyzed preparation) + 25 μmoles DPX + 5 μmoles B_6-PO_4	11,400	0

Rôle of Pyridoxal Phosphate

Nutritional experiments with chicks and bacteria have implicated pyridoxal phosphate as a necessary factor in the biosynthesis of serine. Studies

by Kisliuk and Sakami (15) *in vitro* indicate that the coenzyme is firmly bound to the enzyme molecule, as is the case with the transaminases, with which resolution of apoenzyme from coenzyme has been impossible or extremely difficult (35).

Our studies have shown that the addition of pyridoxal phosphate to the crude extract or lyophilized preparations does not alter the extent of conversion of formaldehyde into serine, indicating that pyridoxal phosphate is tightly bound to the enzyme protein. Attempts were made to decompose pyridoxal phosphate by ultraviolet irradiation. These were suggestive but not conclusive. Other work with an aged lyophilized preparation indicated a slight reactivation by pyridoxal phosphate.

Attention was subsequently turned to the use of the pyridoxal antagonist deoxypyridoxine. Experiments by Umbreit and Waddell (36) elucidated the mechanism of action of this antagonist with the tyrosine decarboxylase of *S. faecalis* and, while our work was in progress, Meister (37) published an account of inhibition of glutamic-aspartic transaminase by deoxypyridoxine phosphate.

Only the dephosphorylated analogue, deoxypyridoxine, was available to us, and it was thought that preincubation of the enzyme preparation with a high concentration of antagonist in the presence of ATP possibly could lead to an inhibition. This suspicion was strengthened since some inhibition was observed when a high concentration of deoxypyridoxine was added at the time of incubation. For reasons at present unknown, much greater inhibition is obtained by preincubating the enzyme preparation with ATP and deoxypyridoxine. A composite of the results reported in Table IX reveals that counteraction of the inhibition by deoxypyridoxine is accomplished only by pyridoxal phosphate, as pyridoxine, pyridoxal, and pyridoxamine are ineffective. Pyridoxal phosphate is more effective when added simultaneously with deoxypyridoxine. One would expect pyridoxal to be phosphorylated and used, but this does not seem to occur.

Similar results were obtained when THFA and the lyophilized preparation were employed (Table IX).

Pyridoxal phosphate has a high affinity for the apoenzyme and can only be replaced when the ratio of analogue to coenzyme is large (Fig. 2, *B*).

DISCUSSION

A comparison of our results with work which has appeared recently yields some interesting differences and similarities.

In the first place, we have observed an activating effect by methionine in addition to the activation by homocysteine reported by Berg (18, 19) and by Deodhar and Sakami (20). This may not be a true difference and the methionine has first to be converted to homocysteine to exert its effect.

The action of homocysteine may be exerted in the conversion of endogenous liver citrovorum factor to THFA or in the interrelationships of the various folic acid derivatives (*cf.* Doctor *et al.* (38)).

Secondly, and more important, none of the work dealing with serine biosynthesis *in vitro* (19, 15–17) has been able to implicate leucovorin, although the biosynthesis of purines from formate requires it. It is plausible that THFA is the active form of the coenzyme required for the utilization of formaldehyde for serine synthesis. Leucovorin is "deformylated" in the presence of ATP to produce THFA by our preparation. In studying purine synthesis, Greenberg (32, 33) reported that ATP reacts with THFA (RH_4) to form a compound he calls RH_4X. This is capable of becoming formylated (FRH_4X) and subsequently used as the "formylation cofactor." Additional work by Nichol (39) also involves ATP and DPN for citrovorum factor synthesis. It is conceivable that a similar system occurs in our preparation. ATP reacts with leucovorin (FRH_4) in the same way to give FRH_4X, which then donates its formyl group to a suitable acceptor (homocysteine(?) or purine precursor) and forms RH_4X or THFA (RH_4) plus X. The THFA thus generated takes part in the conversion of formaldehyde into serine.

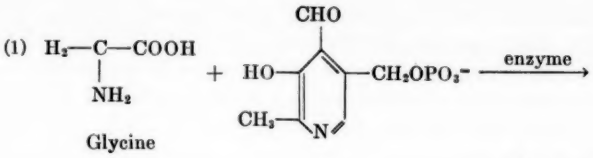
Folic acid will not replace leucovorin in our system nor in that of Greenberg (32, 33), but he has shown that 7,8-dihydrofolic acid plus DPNH can replace THFA. On the other hand, Kisliuk and Sakami (15) and Blakley (17, 40) have reported activation with folic acid. Vilenkina (41) has also reported the reactivation of a dialyzed enzyme preparation by folic acid which catalyzed the formation of formaldehyde and glycine from serine. Blakley (17) has suggested that folic acid is reduced to THFA by formaldehyde (either added or generated in the serine $\rightleftharpoons$ glycine conversion), but, if such were the case, one would expect this to occur in our system with folic acid. Most likely, folic acid is converted to THFA (and citrovorum factor) by DPNH and ATP.

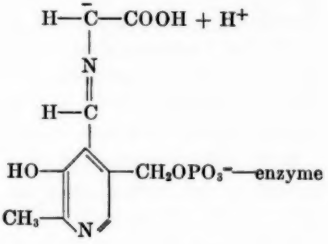
Kisliuk and Sakami (15, 16) and Blakley (17, 40) both worked with pigeon liver extracts. Our work has been done with rat liver. This implies either a difference between the enzymes of the two species or differences in the inactivation or elimination of enzymes by the different methods of preparation and treatment employed.

The rôle of pyridoxal phosphate in the mechanism of serine biosynthesis probably involves the formation of a Schiff base with glycine (15, 23, 42) which activates the methylene carbon atom, as proposed by Metzler *et al.* (42).

The enzymatic mechanism in the accompanying scheme is proposed for the biosynthesis of serine.

Glycine forms the Schiff base with the release of an H^+ ion and the formation of a nucleophilic center at the methylene carbon atom.

(1) 



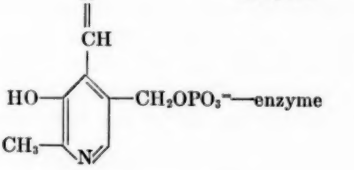
(2) 5-Formyl-5,6,7,8-THFA (leucovorin) + formyl acceptor $\xrightarrow[\text{enzyme}]{\text{ATP}}$

THFA + "formylated product"

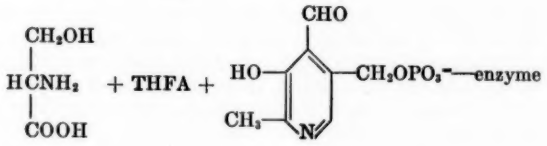
(Folic acid $\xrightarrow{\text{DPNH, ATP}}$ THFA)

(3) $H_2C(OH)_2$* + THFA $\xrightarrow[-\text{SH enzyme}]{}$

5-hydroxymethyl-5,6,7,8-THFA† + H_2O

(4) 5-Hydroxymethyl-5,6,7,8-THFA + 

$\xrightarrow[\text{or enzymatic}]{\text{spontaneous (?)}}$ H_2O, 2H



* Hydrated formaldehyde.

† ($\overset{\delta+}{CH_2}\overset{\delta-}{OH}$)-5,6,7,8-THFA.

When leucovorin or folic acid is added as the cofactor, each must be converted to THFA. This is accomplished respectively with ATP and ATP and DPNH. The formyl group of leucovorin is yielded to a suitable acceptor, *e.g.* a purine precursor or possibly homocysteine, to generate THFA. THFA reacts with formaldehyde to form hydroxymethyl-THFA under the action of an —SH enzyme which may be the same or different from the one containing pyridoxal phosphate.

The final step involves the attraction of the nucleophilic carbon atom of glycine for the electrophilic 1-carbon unit in hydroxymethyl-THFA. This may be spontaneous or enzymatic, and the products are serine, THFA, and pyridoxal phosphate-enzyme.

With THFA as the cofactor, conceivably only one enzyme may be involved in the over-all reaction (this enzyme would have pyridoxal phosphate firmly bound to it). With leucovorin and ATP as the source of the cofactor, at least two and possibly more enzymes are required. Fractionation studies should help to elucidate this.

SUMMARY

1. A cell-free, soluble rat liver enzyme has been obtained that anaerobically catalyzes the synthesis of serine from glycine and formaldehyde. The breakdown of serine to glycine and formaldehyde by the preparation is slight in comparison.

2. Methionine or homocysteine, ATP, and Mg^{++} are activators in the conversion. The action of methionine is probably mediated through its ability to form homocysteine. ATP is probably involved in the conversion of endogenous citrovorum factor to THFA.

3. A significant finding is the loss of activity by dialysis, which can be restored by ATP and leucovorin together or by THFA alone.

4. Preincubation with ATP and deoxypyridoxine inhibits the system. The inhibition is reversed by pyridoxal phosphate.

5. Studies with hydrogen peroxide and *p*-chloromercuribenzoate show the enzyme to require —SH groups for activity.

6. A general mechanism for the biosynthesis of serine is presented.

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XUM

THE SPECIFIC AMINO ACID REQUIREMENTS OF A MAMMALIAN CELL (STRAIN L) IN TISSUE CULTURE

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PLATE 1

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There have been many attempts to devise chemically defined media which will permit the propagation of mammalian cells in tissue culture, without supplementation by serum, plasma, or tissue extractives. To date, these attempts have not been successful. Although some of these media, notably those recently developed by Morgan, Morton, and Parker (1) and by Healy, Fisher, and Parker (2), permit the survival of some cell lines for long periods, in our hands none has allowed continuing multiplication on serial subculture. Growth can be obtained on the addition to such media of a small amount of serum, but the medium is then no longer chemically defined and no longer permits the delimitation of the essential growth requirements of the cell.

Recognizing this difficulty, Fischer (3) had used the expedient of supplementing a defined medium with dialyzed chicken plasma, serum, and embryo extract, in the hope that the essential small molecular weight components of the defined medium could then be identified. On the omission of either cystine, arginine, tryptophan, glutamine, or histidine and proline, there was in fact a decrease in the amount of radial growth from a chick embryo myoblast explant. However, growth was not prevented, and no single component could be rigorously characterized as essential to survival and growth. In the light of the data to be reported here, it is possible (*a*) that the culture materials used by Fischer were not adequately dialyzed, (*b*) that the large amounts of plasma and serum employed (more than 50 per cent by volume) contained compounds which could substitute in part for the amino acids, or (*c*) that the diameter of radial growth around the explant was not a true measure of cell proliferation. In any event, in the simpler system used here, in which cells were grown on a glass surface, the addition of small amounts of dialyzed horse serum to an otherwise defined medium permitted long term propagation of the adult mouse fibroblast (L strain). In such a limiting medium, the omission of a single essential growth factor now resulted in the early death of the culture, and it proved

possible to define, both qualitatively and quantitatively, the specific amino acid requirements of this cell line.

Subsequent papers will describe the specific amino acids essential for other cell lines, their minimal vitamin and salt requirements, the important rôle of glutamine, and the function of the serum protein.

Methods

Technique of Cultivation—The cell line used in these experiments was the L strain of mouse fibroblast cultured from a single cell by Sanford, Earle, and Likely (4). When a suspension of these cells is placed in a flat bottomed flask, the cells sediment and adhere to the glass, where they grow out as a monocellular layer which eventually covers the glass surface (5). Stock cultures were grown in a medium consisting of 6 per cent horse serum,[1] 20 per cent chick (or beef) embryo extract ultrafiltrate,[1] 73 per cent Earle's balanced salt solution, and 1 per cent of an antibiotic mixture containing 5 mg. per ml. of penicillin, 5 mg. per ml. of streptomycin, and 0.5 mg. per ml. of phenol red.[1] The stocks were maintained in 1 liter Blake bottles containing 40 to 50 ml. of medium. The experimental flasks were the T-15 flasks described by Earle and Highhouse (6), modified by placing the neck on the triangular instead of the flat end of the flask. For the experiment, a 7 to 10 day Blake bottle culture (approximately 10 to 20×10^6 cells) was scraped into, for example, 100 ml. of the stock medium, the cell count was determined (7), and the appropriately diluted suspension distributed in 2 ml. amounts into the experimental flasks. (The size of the inoculum, *i.e.* the number of cells introduced into the flask, as well as the number which had adhered to the glass at the time of the first feeding, was determined on four to eight flasks and varied in early experiments from 80,000 to 250,000 cells per flask. In later experiments, the inoculum was standardized at 200,000 to 250,000 cells.) 24 hours later, after the cells had adhered to the glass, the fluid was withdrawn, the flasks were washed with 1 ml. of the various experimental media (containing the components of Table I in varying combination supplemented with 0.5 to 2 per cent of dialyzed horse serum), and 2 ml. added for actual incubation.

The medium was replaced after 24 hours at 37° and again at 2 to 3 day intervals until a solid sheet had formed. With "complete" media this usually required 5 to 8 days. The cell count was then determined, and the culture flasks either discarded or divided. For division, the supernatant fluid was withdrawn, 4 ml. of fresh medium were added, and the cells scraped off the glass into the fresh medium. 2 ml. of the suspension were then plated into a fresh flask, and the remainder was used for the cell count. The technique of cell enumeration was based on that described by Sanford

[1] Purchased from Microbiological Associates, Bethesda, Maryland.

et al. (7). 0.4 ml. of the cell suspension was added to 0.4 ml. of a solution containing 0.2 M citric acid and 0.02 per cent crystal violet. The discrete nuclei were sufficiently stained for counting in a hemocytometer within 10 to 15 minutes.

TABLE I

Basal Medium Used for Identification of Essential Growth Requirements of Mouse Fibroblast (L Strain)*

Amino acids (L- or DL-), 0.1 to 1 mM	Vitamins, 10^{-6} to 10^{-8} gm. per ml.	Inorganic salts†	Additional growth factors, mM
Alanine	*p*-Aminobenzoic acid	NaCl	Choline 0.005
Arginine‡	Ascorbic acid	KCl	Glucose 5.5
Aspartic acid	Biotin	NaH_2PO_4	Glutamine 0.5
Cystine‡	Folic acid	$NaHCO_3$	Glutathione 0.1
Glutamic acid	Nicotinic acid	$CaCl_2$	Inositol 0.1
Glycine	Nicotinamide	$MgCl_2$	
Histidine‡	Pantothenic acid	$Fe(NO_3)_3$	
Hydroxyproline	Pyridoxal		
Isoleucine‡	Pyridoxine		
Leucine‡	Riboflavin		
Lysine‡	Thiamine		
Methionine‡	α-Tocopherol		
Phenylalanine‡	Vitamin A		
Proline	" D		
Serine	" K		
Threonine‡			
Tryptophan‡			
Tyrosine‡			
Valine‡			

* Supplemented with 0.25 to 2 per cent of dialyzed horse serum. A number of the components used in various "synthetic media" proved unnecessary for the growth of the L strain and are not listed above: purines, pyrimidines, acetate, pyruvate, coenzyme A, adenylic acid, adenosine triphosphate, diphosphopyridine nucleotide, triphosphopyridine nucleotide. Further, as will be shown in the present and following papers, almost half of the components listed in the table ultimately proved to be non-essential.

† Concentrations as provided in Earle's balanced salt solution (8). The $Fe(NO_3)_3$ was used at a final concentration of 10^{-7}.

‡ Here shown to be essential for survival and growth.

Results

Identification of Essential Amino Acids—In a medium such as that outlined in Table I, containing all the amino acids in concentrations of 0.1 to 1 mM, a number of vitamins at concentration ranges of 10^{-6} to 10^{-8} gm. per ml., and arbitrarily selected accessory growth factors and inorganic salts, the cells often survived for days, but eventually, and particularly after

TABLE II

Twelve Amino Acids Essential for Survival and Multiplication of L Strain Mouse Fibroblast and Seven Non-Essential

Amino acid omitted from medium	Inoculum × 10^4	Incubation period, days	Final cell count × 10^{4*}	Degree of proliferation*†	Microscopic appearance of cells
0 (Complete medium, control)	9	7	39	4.3	Healthy, spindle-shaped, and stellate cells, grown out to form solid sheet on surface of glass
	9	8	30	3.3	
	18	8	71	4.0	
	18	11	72	4.0	
	22	8	90	4.9	
	25	7	149	6.0	
Arginine	21	5	9	0.4	Discrete cells dead or degenerating; many floating in medium; those still adherent were pyknotic, granular, or vacuolated; nucleus count fell off progressively on continued incubation
	13	6	8	0.6	
	8	8	7	0.9	
	22	7	7	0.3	
Cystine	9	4	3	0.3	
	18	8	21	0.1	
Histidine	21	5	6	0.3	
	13	6	5	0.4	
	18	8	7	0.4	
	22	8	9	0.4	
Isoleucine	13	11	4	0.3	
	18	8	2	0.1	
	21	7	8	0.4	
Leucine	21	5	8	0.4	
	13	6	7	0.6	
Lysine	13	6	6	0.5	
	9	4	2	0.2	
	22	8	7	0.3	
Methionine	21	5	6	0.3	
	13	6	4	0.3	
	18	8	7	0.4	
	25	7	6	0.2	
Phenylalanine	21	5	6	0.3	
	13	6	5	0.4	
	18	8	7	0.4	
	22	6	6	0.3	
Threonine	21	5	4	0.2	
	13	6	3	0.2	
	18	8	4	0.2	
Tryptophan	21	5	8	0.4	
	13	6	4	0.3	
	18	8	6	0.3	
Tyrosine	9	4	2	0.2	
	16	9	1	0.06	
Valine	13	6	3	0.2	
	9	4	2	0.2	

TABLE II—*Concluded*

Amino acid omitted from medium	Inoculum × 10^4	Incubation period, days	Final cell count × 10^{4*}	Degree of proliferation*†	Microscopic appearance of cells
Alanine	9	7	20	2.2	Healthy, spindle-shaped, and stellate cells, grown out to form solid sheet on surface of glass
	18	8	46	2.6	
Aspartic acid	9	15	55	6.1	
	18	8	66	3.7	
Glutamic acid	9	7	32	3.6	
	13	7	58	4.5	
Glycine	9	7	32	3.6	
	13	7	48	3.7	
Hydroxyproline	18	8	48	2.7	
	13	7	54	4.1	
Proline	9	15	61	6.8	
	18	15	44	2.4	
Serine	21	5	53	2.5	
	13	6	44	3.4	
	18	8	87	4.8	

* Each figure listed represents a different experiment, and each is the average of two to five experimental flasks.

† Cell count after incubation is here referred to the inoculum as 1. Numbers less than 1 reflect degeneration and lysis of the inoculum.

attempts to subdivide such cultures into fresh medium, the cells rapidly degenerated and died. However, when this chemically defined mixture was supplemented with 0.5 to 2 per cent dialyzed horse serum, with no other serum or tissue derivative, the cells not only survived, but grew out on the surface of the glass to form a continuous sheet within a period of 5 to 8 days (see Fig. 1). The average generation time under the conditions of the present experiments varied from 2 to 3 days. On subdivision, the cells again grew out, and in a number of experiments growth in this medium was maintained for periods of 4 to 8 weeks with no apparent change in the rate of growth or in the appearance of the cells. Although not discussed in the present paper, it is to be emphasized that the multiplication of the cells as measured by nucleus counts was paralleled by an increase in total cell volume, cell mass, and protein N.

If, from such a medium, one omitted a single essential amino acid, there was no significant multiplication. After a period which varied from 1 to 3 days, depending on which amino acid has been omitted, obvious degenerative changes occurred, and the cultures soon died (Fig. 2). In this manner, twelve amino acids (arginine, cysteine (cystine), histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine) were shown to be essential for survival and growth (see upper portion of Table II).

XUM

TABLE III

Degree of Cell Multiplication As Function of Amino Acid Concentration in Medium

Amino acid studied*	Inoculum × 10^4	Duration of incubation, days	Concentration of specific amino acid,* μmoles per ml.												
			5	2	0.5	0.2	0.1	0.05	0.02–0.025†	0.01–0.0125†	0.005–0.006†	0.002–0.0025†	0.001–0.0012†	0.0005–0.0006†	0
			Degree of cellular multiplication,‡ referred to inoculum as 1												
L-Arginine	22	7		3.2	3.5	4.1	3.7	4.1	3.0†	2.1†		0.5	0.5		0.3
	25	6	6.4	6.0	6.3		6.1								
L-Cystine	24	6		4.2	5.4	4.6	4.7	4.9	5.3†	4.5†	2.8†	1.1†			0.1
	25	6	4.6	5.5	4.8		5.4								
L-Histidine	22	8		4.2	4.7	4.5		4.4	4.9	4.4	3.0	1.7†	1.0†		0.4
	25	6	5.4	6.4	7.4				6.1†						
L-Isoleucine	21	7		3.9	6.0	5.7	5.2	3.7	2.3	1.3	0.7	0.4†	0.35†		0.4
	25	6	4.5	7.2	6.1	5.4									
L-Leucine	20	6		6.2	8.0	6.1	7.3	6.0	3.2	2.0	1.4	1.2†	1.0†		1.0
	25	6	4.7	5.0	4.9		5.4								
L-Lysine	22	8					3.1	3.4	2.6†	0.9†	0.36†				<0.1
	25	6	6.8	6.6	6.0		6.1								
L-Methionine	25	7		1.0	3.8	4.4		6.0	5.4	3.1†	1.64		0.7†		0.3
	25	6	0.52	0.7	2.5			6.1							
L-Phenylalanine	22	6		4.0	4.6		4.6	4.2	4.3†	3.0	1.4	0.6†			0.3
	25	6	3.4	6.6	6.6			6.1							
L-Threonine	29	8		3.0	3.7	3.6	4.2	3.4	3.1†	1.4†	0.6†	0.3†			0.1
	25	6	7.0	6.8	6.7		5.4								
L-Tryptophan	22	7						2.6	4.6†	3.5	4.3	2.4†	1.7†	1.0†	<0.1
	25	7		2.4	2.9	2.6		2.7	4.8		5.0	1.8		0.44	0.4
	25	6	2.4	4.5	5.6					6.1					
L-Tyrosine	24	6		4.7	5.6	4.5	5.1	5.1	4.4	3.0	1.25	0.6†			0.35
	25	6	4.8	5.1	4.9		5.4								
L-Valine	24	6		4.2	4.4	4.5	4.8	4.5	1.75	1.0	0.46	0.35†			0.15

L-Valine	24 25	6 6	6.8	4.2 6.2	4.4 6.6	4.5	4.8 5.4	4.5	1.75	1.0	0.46	0.35†			0.15

* In a medium containing the other eleven essential amino acids at "optimal" concentrations (see Table IV).

† The readings carrying this symbol were obtained at the higher concentration.

‡ The differences in the degree of cell multiplication observed in different experiments reflect the progressive improvement in the medium as the optimal level of the various components was more precisely defined, as well as experimental variation.

Under the conditions of the present experiments, seven amino acids (alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, proline, and serine) were not essential (lower portion of Table II). In their absence the cells continued to proliferate after repeated subdivisions over a period of at least 6 weeks. It is unlikely that the 0.05 to 0.1 per cent of serum protein in the medium was supplying these seven amino acids. Were the protein being broken down sufficiently to furnish adequate amounts of those apparently non-essential compounds, it would reasonably be expected to supply at least some of the other twelve, particularly since two of

TABLE IV

Effective Concentrations of Each of Twelve Amino Acids Required for Growth of L Strain Fibroblast in Tissue Culture

Amino acid (L configuration)	Minimal concentration permitting limited growth	Concentration permitting maximal growth	Concentration in mouse plasma*
	mM	mM	mM
Arginine	0.01	0.05	0.056
Cystine	0.005	0.01	0.033
Histidine	0.002	0.01	0.10
Isoleucine	0.01 –0.02	0.1 –0.2	0.12
Leucine	0.005 –0.01	0.05	0.18
Lysine	0.02	0.05	0.44
Methionine	0.005	0.02 –0.05	0.13
Phenylalanine	0.005 –0.01	0.02	0.15
Threonine	0.01 –0.02	0.05	0.3
Tryptophan	0.00125	0.005	0.10
Tyrosine	0.005 –0.01	0.02 –0.05	0.14
Valine	0.02	0.05	0.37

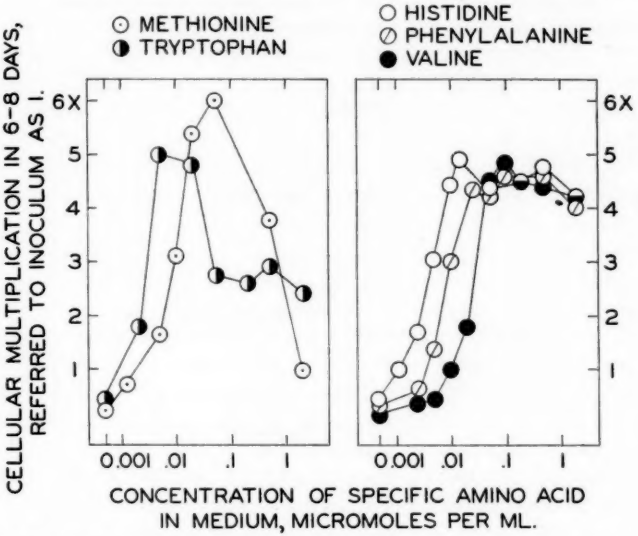
* After Albritton (11).

the latter were growth-promoting in a concentration of 0.001 μmole per ml. Further, it is to be noted that the seven amino acids not required for growth under the conditions of the present experiments are similarly non-essential for the whole animal (9, 10).

Microscopic Changes Produced by Specific Amino Acid Deficiencies and Reversal on Restoration of Missing Component—Profound changes occurred in the microscopic appearance of the cells when any one essential amino acid was omitted from the medium. These are illustrated in Fig. 2 and will be described in detail in a following communication. For the first 2 to 6 days, these cytopathologic effects of an amino acid deficiency were completely reversible. On the addition of the missing amino acid to the medium, normal cellular structure was reestablished within 1 to 3 days. The damaged cells then resumed multiplication, and the culture became mor-

phologically indistinguishable from control, untreated cells. This revival of cells suffering from a specific amino acid deficiency is illustrated for one compound, lysine, in Fig. 4.

Effective Concentrations of Twelve Essential Amino Acids—The effective concentrations of each of the twelve essential amino acids were now determined by measuring the amount of cell proliferation within 6 to 8 days in media containing fixed amounts of eleven amino acids and varying con-



TEXT-FIG. 1. Illustrating two types of growth response to amino acid. In the left-hand section, methionine and tryptophan showed a well defined optimal range, with inhibition of growth in excess of that optimum. With the three essential amino acids (shown in the right-hand section), concentrations greatly in excess of the maximally effective level had no significant effect on the rate of multiplication.

centrations of the twelfth. In these experiments, all the compounds were in the L configuration. A typical experiment is illustrated in Fig. 3, which shows the striking differences in the microscopic appearance of the cultures as the concentration of a single amino acid was varied. The results of a number of similar experiments are summarized in Tables III and IV and in Text-fig. 1.[2]

[2] The difference in the maximal degree of cell proliferation observed in individual experiments is of no significance. In large part, it reflects the progressive modification in the composition of the medium as the work progressed and as the optimal level of the various components was more precisely defined.

With each of the twelve amino acids there was a well defined maximally effective concentration, which varied from 0.005 μmole per ml. in the case of tryptophan to 0.1 to 0.2 μmole per ml. in the case of isoleucine (see Table IV). Although the absolute requirements for the individual amino

TABLE V

Showing That D *Enantiomorphs of Essential Amino Acids Do Not Support Growth of L Fibroblast; nor Do They Inhibit Growth Response to* L *Isomer*

Amino acid	Concentration in medium of		Growth response* in 6-8 days	Amino acid	Concentration in medium of		Growth response* in 6-8 days
	D-	L-			D-	L-	
	mM	mM			mM	mM	
Arginine	1		0.42†	Methionine	1		0.3
	0.1		0.45		0.1		0.45
		0.1	3.7			0.1	4.4
	1	0.1	2.9		1	0.1	4.7
Cystine	1		0.4	Phenylalanine	1		0.8
	0.1		0.35		0.1		0.6
		0.1	4.7			0.1	4.6
	1	0.1	3.8		1	0.1	3.8
Histidine	1		0.45	Threonine	1		0.15
	0.05		0.41		0.1		0.1
		0.01	4.4			0.1	4.2
	1	0.01	3.8		1	0.1	3.3
Isoleucine	1		1.05	Tryptophan	0.01		0.64
	0.1		0.24			0.01	3.5
		0.1	5.2		0.1	0.01	2.7
	1	0.1	5.5				
Leucine	1			Tyrosine	1		0.33
	0.1				0.1		0.25
		0.1	7.3			0.1	5.1
	1	0.1	8.2		1	0.1	4.3
Lysine	0.33		0.17	Valine	1		0.15
	0.1		0.33		0.1		0.12
		0.1	3.1			0.1	4.8
	0.33	0.1	2.9		1	0.1	5.1

* Referred to inoculum as 1.

† Numbers less than 1 indicate that the cells had degenerated and lyzed, instead of multiplying.

acids thus varied 20-fold, the shapes of the dosage-response curves at suboptimal concentrations were remarkably uniform (left-hand portion of Text-fig. 1). With most of the compounds, one-half to one-fifth of the maximally effective concentration barely permitted survival and limited multiplication, and, at concentrations intermediate between that minimal maintenance level and the concentration required for maximal growth, there was a progressive and uniform increase in the rate of cell multiplication.[2]

At concentrations in excess of the maximally effective level, however, the amino acids fell into several groups, as illustrated in Text-fig. 1. With methionine and tryptophan there was a sharply defined optimal level at 0.05 and 0.005 to 0.02 μmole per ml., respectively. Concentrations in excess of that optimum caused a marked and progressive inhibition in the rate of cell multiplication (left-hand portion of Text-fig. 1). With isoleucine and phenylalanine there was slightly decreased growth at a concentration of 5 μmoles per ml. With all the remaining compounds, however, concentrations as high as 5 μmoles per ml., 50 to 500 times those which provided for maximal growth, had no significant effect on the rate of multiplication (see Table III and right-hand section of Text-fig. 1).

Relative Growth-Promoting Activity of D- *and* L-*Amino Acids*—None of the twelve essential L-amino acids could be replaced by their D enantiomorphs. Commercial samples of D-leucine, D-methionine, and D-phenylalanine did have a growth-promoting activity 5 to 10 per cent that of the L isomer. However, pure samples obtained by enzymatic resolution (12), generously made available by Dr. Jesse Greenstein, proved inactive, and the weak growth-promoting activity of the commercial product was therefore referable to contamination with the L isomer.

Although the D-amino acids did not support growth, none was actively growth-inhibitory under the conditions of the present experiments. Media containing the essential L-amino acids at optimal levels, plus a single D-amino acid at 5 to 10 times the concentration of the corresponding L isomer, were just as active in supporting growth as the mixture containing the L-amino acid alone (Table V).

DISCUSSION

The present experiments describe the specific amino acid requirements in tissue culture of the single cell L strain of adult mouse fibroblast. It is of interest that the requirements were qualitatively greater than those of the intact animal. Nine amino acids have been described (9, 10) as essential for the adult mouse (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine). Not only were these nine compounds essential for the growth of the mouse fibroblast *in vitro*,

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but in addition arginine, cysteine (or cystine), and tyrosine. The isolated cell thus had a triple requirement for phenylalanine, tyrosine, and tryptophan and, similarly, needed both cystine and methionine.

It is possible that the cell under present study can in fact synthesize the three essential amino acids which are not similarly essential to the parent animal (arginine, cystine, tyrosine), but only at a slow rate. That slow synthesis might suffice for cellular multiplication *in vivo*, but would be inadequate for the much greater rate of growth observed *in vitro*. It is further possible that the nutritional requirements of this cell line have been profoundly modified in the course of 6 years of cultivation *in vitro*. However, if neither of these possibilities is correct, and if the growth requirements of mouse fibroblasts *in vivo* are qualitatively the same as those here found *in vitro*, it would follow that, on a diet containing only the nine amino acids essential for the adult mouse, other body cells elaborate the additional three amino acids required by this fibroblast, or utilizable precursors of those acids. If this proves to be a general phenomenon, if cells of varying function do in fact differ materially in their nutritional requirements, then different cell types, even within a single organ or tissue, may significantly affect each other's growth rates. Further, in studying the metabolic activities of tissue slices or homogenates, one would be measuring the composite and possibly qualitatively varying activities of a number of different cell types.

One of the most striking aspects of the present studies was the complete arrest of cell growth and multiplication on the omission of a single essential amino acid from the medium, the marked changes which then occurred in the microscopic appearance of the cells, and the complete reversal of that cytopathogenic effect if the missing amino acid was restored to the medium within a period of 2 to 6 days. The metabolic activities of the cell which permit the utilization of the essential amino acids thus remain functional despite the marked cytopathologic changes caused by an amino acid deficiency. Studies are now in progress to determine whether a single amino acid deficiency prevents protein and nucleic acid synthesis as effectively as it prevents cellular growth and multiplication, or whether there is a continuing formation of new protein and nucleic acid at the expense of preexisting cell components.

The failure of any of the D isomers of the twelve essential amino acids to support the growth of the cells studied here is in keeping with the fact that D-amino acid oxidases have to date been demonstrated only in liver or kidney (references cited by Meister (13)).

Many explanations have been suggested for the fact that cells in culture usually multiply at a rate greatly exceeding their estimated rate of growth *in vivo*. One possibility is that one or more essential metabolites are present in the body fluids in either growth-limiting or growth-inhibitory

concentrations. With respect to the former, the present experiments indicate that, at least for the cell studied here, the concentrations in mouse plasma of ten of the essential amino acids are significantly in excess of those necessary for growth at a maximal rate *in vitro* (see Table IV). With arginine and isoleucine, however, the concentrations in the plasma are sufficiently close to those needed by the cell for optimal growth to suggest that the concentrations in the tissue fluids might in fact be growth-limiting (Table IV). As to growth inhibition, under the conditions of the present experiments even a large excess of the essential amino acids (up to 5 μmoles per ml.) failed to inhibit growth significantly, except in the case of methionine and tryptophan. With methionine, the plasma concentration of 0.13 μmole per ml. was well below the growth-inhibitory level of 0.5 μmole per ml., but with tryptophan, the plasma concentration of 0.1 μmole per ml. sufficed to cause a significant diminution of growth in two of three experiments (see Table III).

SUMMARY

Mouse fibroblasts (L strain) were grown on a medium consisting of a limited number of chemically defined compounds, supplemented with 0.5 to 2 per cent of dialyzed horse serum.

1. On such a medium, twelve amino acids in the L configuration proved to be essential for growth (arginine, cysteine (cystine), histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine), and seven proved non-essential (alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, proline, and serine). It is of interest that three of the essential compounds (arginine, cystine, and tyrosine) are not essential for the growth of the whole animal.

2. In the absence of any one of the essential amino acids, there was no multiplication, and the cells degenerated and eventually died. During the first 2 to 6 days, however, the cytopathologic changes caused by a single amino acid deficiency could be reversed by restoring the missing compound to the medium, and the recovered cells then resumed multiplication at a normal rate.

3. The concentration of the individual amino acids permitting growth at a maximal rate varied 20-fold, from 0.005 μmoles per ml. in the case of tryptophan to 0.1 to 0.2 μmole per ml. for isoleucine. At lower concentrations, the rate of multiplication fell off sharply. At higher concentrations, even 50 to 500 times the maximally effective level had no significant effect, except in the case of tryptophan and methionine. With these, concentrations of 0.05 and 0.5 μmole per ml., respectively, caused a distinct retardation of growth.

4. None of the D isomers of the essential amino acids supported growth, but none inhibited the growth-promoting effect of the L isomer.

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The assistance of Ralph Fleischman, Mina Levy, and Clara Horton in the performance of these laborious and repetitive experiments is gratefully acknowledged.

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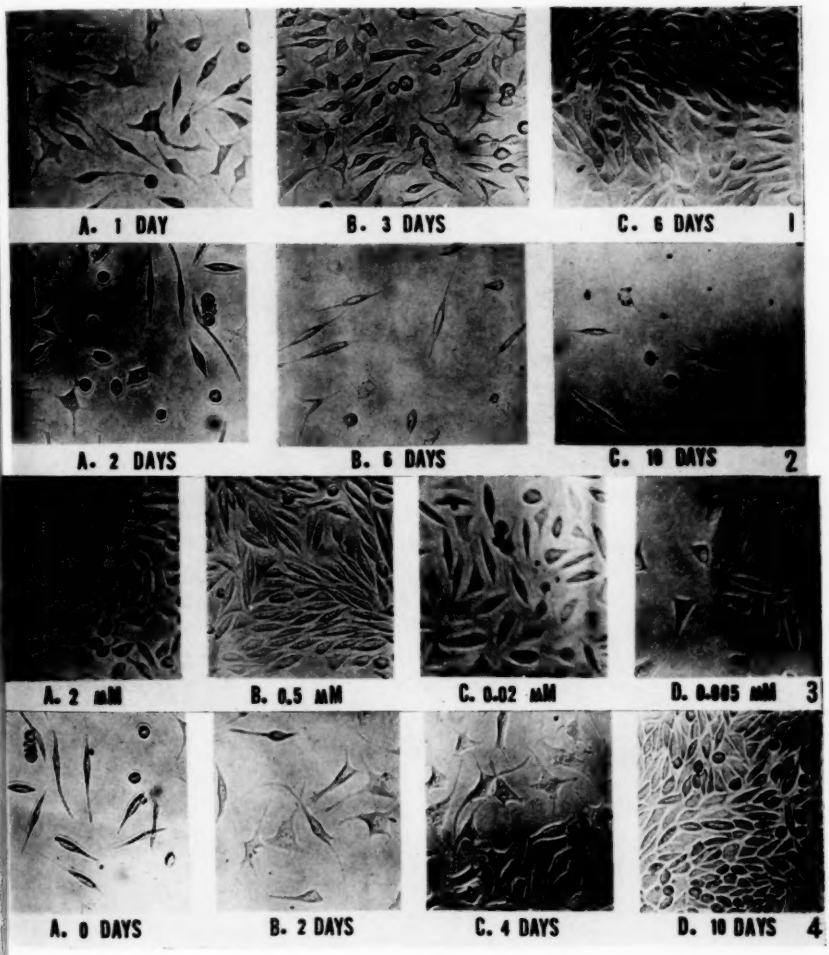
EXPLANATION OF PLATE 1

FIG. 1. The growth of a mouse fibroblast (L strain) in a "complete" medium (Table I). × 100.

FIG. 2. Illustrating the death of the L strain in a medium deficient in a single amino acid (L-lysine). × 100.

FIG. 3. Illustrating the growth response of the L strain as the concentration of a single essential amino acid (isoleucine) was varied, all other components in the medium remaining constant. × 100.

FIG. 4. Illustrating the recovery of the L strain from a specific amino acid deficiency (L-lysine) when the missing component was restored to the medium in optimal concentration. Cells, which had been exposed to a lysine-deficient medium for 2 days and which were largely degenerated in consequence, resumed their normal appearance within 2 days and went on to multiply at a normal rate. × 100.



(Eagle: Amino acid needs of mammalian cells)

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THE OCCURRENCE AND MECHANISM OF THE HEXOSE MONOPHOSPHATE SHUNT IN RAT LIVER SLICES*

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It was recently shown by Bloom *et al.* (1, 2) that, in liver, the 1st carbon of glucose is converted to CO_2 to a larger extent than is the 6th carbon, and that the $C^{14}O_2$ derived from glucose-1-C^{14} exceeds that from glucose evenly labeled with C^{14}. These workers suggested that the hexose monophosphate shunt plays a significant rôle in glucose metabolism in liver. A previous report from this laboratory dealt with equations for estimating the extent of the hexose monophosphate shunt in that tissue (3). In the present report, those equations have been extended, and the assumptions upon which they are based have been examined. The operation of this shunt in liver slices was studied, and some phases of its mechanism have been elucidated.

Materials and Methods

Preparation of Labeled Substrates—Glucose evenly labeled with C^{14} (glucose-E-C^{14}) was prepared photosynthetically as described by Putman and Hassid (4). Glucose-1-C^{14} and ribose-1-C^{14} were purchased from the National Bureau of Standards. Glucose-6-C^{14} was prepared by the method of Sowden (5) as modified by Roseman (6). All sugars were checked chromatographically for purity.

Incubation Procedure—Long-Evans rats weighing from 200 to 300 gm. were fed a high carbohydrate diet (7) for 3 days before they were sacrificed. The preparation of liver slices and the incubation techniques were described in previous reports (8).

Isolation of C^{14}-Glucose from Incubation Mixture and Determination of C^{14} Content of Its Individual Carbon Atoms—Glucose-C^{14} was isolated chromatographically on paper (8) and eluted with water. Carrier glucose was added, and the solutions were concentrated. The C^{14}-glucose was degraded by fermentation with *Leuconostoc mesenteroides* which yields CO_2 from carbon 1, ethanol from carbons 2 and 3, and lactate from carbons 4, 5, and 6 (9). The organisms were grown for 12 hours and harvested essentially as described by Gunsalus and Gibbs (9). The organisms were washed three times and tested for activity before use.

* This work was supported by a contract from the United States Atomic Energy Commission.

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The type of flask used for fermentation has been described elsewhere (10). The fermentation mixture consisted of the cells from about 1 liter of growth medium, 0.4 mmole of the C^{14}-glucose, and 5 ml. of 0.5 M phosphate buffer (pH 6.0) in a final volume of 12 to 15 ml. The vessels were flushed with nitrogen, alkali was added to the center wells, and the vessels were capped and evacuated with a hypodermic needle. The fermentation was terminated after 1 hour, and the organisms were removed by centrifugation. The recovery of CO_2 was quantitative, but that of ethanol and lactate ranged from 60 to 70 per cent. The ethanol was obtained by distillation of about one-half the volume of the neutralized medium, then oxidized to acetate with acid-dichromate (11), and the acetate was recovered by steam distillation. The non-volatile fraction containing the lactate was acidified to pH 2, excess silica was added, and the fairly dry mixture was extracted on a Büchner funnel with a 1:1 mixture of wet *n*-butanol-chloroform. In some experiments, anhydrous sodium sulfate was used instead of silica, and the dry mixture was extracted with ether. Water was added to the extract, the lactate was then titrated with base, and the organic solvents were evaporated. The methods used for the degradation of lactate and acetate were described elsewhere (12).

When glucose-1-C^{14}, -2-C^{14}, and -6-C^{14} were degraded by this procedure, about 2 to 5 per cent of the C^{14} was recovered in carbons other than 1, 2, and 6. This may be due in part to some side reactions of bacterial metabolism; hence, incorporation of the order of 5 per cent of the C^{14} in any 1 carbon is not considered significant.

Evaluation of Hexose Monophosphate Shunt in Liver Slices

The conversions of glucose-1-C^{14}, -6-C^{14}, and -E-C^{14} to $C^{14}O_2$ are compared in Table I. In each experiment, separate portions of a single liver were incubated with either glucose-1-C^{14}, -6-C^{14}, or -E-C^{14}. When glucose served as the sole substrate, the $C^{14}O_2$ yields ranged from 2 to 13 per cent for glucose-1-C^{14}, from 0.7 to 4.5 per cent for glucose-6-C^{14}, and from 2 to 5 per cent for glucose-E-C^{14}. It should be noted, however, that, *in the case of each liver*, the $C^{14}O_2$ recoveries from glucose-1-C^{14} were about the same as that from glucose-E-C^{14} and were appreciably higher than those observed with glucose-6-C^{14}. This is reflected in the values for the ratio, U, *i.e.* ($C^{14}O_2$ from glucose-E-C^{14})/($C^{14}O_2$ from glucose-1-C^{14}), which ranged from 0.7 to 1.2 (except for a single value as high as 2), and the ratio, W, *i.e.* ($C^{14}O_2$ from glucose-6-C^{14})/($C^{14}O_2$ from glucose-1-C^{14}), which ranged from 0.33 to 0.65.

The values for ratios U and W observed here in experiments in which glucose served as the *sole* substrate were higher than those reported by Bloom *et al.* (1, 2). These investigators, however, added 50 μmoles each of lactate, acetate, and gluconate to their incubation media. Table I

TABLE I

Oxidation of Variously Labeled C^{14}-Glucose

250 mg. of liver slices were incubated in 2.5 ml. of Ringer-bicarbonate buffer at pH 7.3 (24) containing (unless otherwise stated) 55 μmoles of labeled glucose. The addition referred to by + was a mixture of 50 μmoles each of acetate, lactate, and gluconate as their Na salts. Gas phase 95 per cent O_2-5 per cent CO_2. 3 hours incubation at 37°.

Rat No.	Label in glucose	Addition to medium	Per cent of added C^{14} recovered as $C^{14}O_2$	$U = \frac{C\text{-}E}{C\text{-}1}$	$W = \frac{C\text{-}6}{C\text{-}1}$	Contribution of glycolysis to	
						CO_2 formed from glucose (E)*	Glucose catabolized (F)†
						per cent	*per cent*
3	C-E		5.0	2.0		98	94
	C-1		2.5				
4	C-E		4.1	0.95		88	70
	C-1		4.3				
5	C-E		4.8	1.02		89	72
	C-1		4.7				
6	C-E		4.6	0.98		89	72
	C-1		4.7				
7	C-E		3.3	0.87		86	67
	C-1		3.8				
8	C-E		2.9	0.81		85	64
	C-1		3.6				
	C-E	+	0.8	0.31		47	23
	C-1	+	2.6				
9	C-E		2.9	0.76		83	61
	C-1		4.2				
	C-E	+	1.0	0.37		57	31
	C-1	+	2.7				
10	C-E		2.8	1.2		92	78
	C-1		2.3				
	C-E	+	0.8	0.57		76	49
	C-1	+	1.4				
11	C-E		4.5	1.2		89	72
	C-1		4.4				
	C-E	+	0.7	0.32		50	25
	C-1	+	2.2				
16	C-6		4.0		0.36	89	75
	C-1		11.2				
	C-6	+	1.9		0.12	69	42
	C-1	+	10.3				
17	C-6		4.5		0.36	90	75
	C-1		12.3				
	C-6	+	1.5		0.14	73	45
	C-1	+	10.9				
18	C-6		3.4		0.65	97	91
	C-1		5.3				
	C-6	+	1.2		0.21	81	58
	C-1	+	5.3				

TABLE I—*Concluded*

Rat No.	Label in glucose	Addition to medium	Per cent of added C^{14} recovered as $C^{14}O_2$	$U = \frac{C\text{-}E}{C\text{-}1}$	$W = \frac{C\text{-}6}{C\text{-}1}$	Contribution of glycolysis to	
						CO_2 formed from glucose (E)*	Glucose catabolized (F)†
						per cent	*per cent*
19	C-6		5.1		0.39	91	77
	C-1		13.1				
	C-6	+	3.0		0.18	78	54
	C-1	+	17.7				
20‡	C-E		2.0	0.96		88	71
	C-6		0.7		0.33	88	71
	C-1		2.1				
21‡	C-E		3.3	0.69		80	57
	C-6		1.6		0.33	88	71
	C-1		4.8				

* Calculated from Equation 1 or 2 with $R = 2.7$.

† Calculated from Equation 3 or 4 with $R = 2.7$ and $P = 3.1$.

‡ In the experiments carried out with the livers of these rats, only 3 μmoles of labeled glucose were incubated in 2.5 ml. of the medium with 250 mg. of slices.

shows that, when these additions are made, there is a lowering of the values for both ratios. It should be noted, however, that the concentrations (0.02 M) of lactate, acetate, and gluconate used by us were twice those used by Bloom *et al.* (1, 2) and that the CO_2 ratios obtained by us upon the addition of these compounds were lower than the ones reported by Bloom *et al.* These additions considerably depressed the $C^{14}O_2$ yields from glucose-6-C^{14} and glucose-E-C^{14}, but had little or no effect on the $C^{14}O_2$ yields from glucose-1-C^{14}.

In order to evaluate the relative significance of the glycolytic and non-glycolytic pathways of glucose metabolism in rat liver, Bloom *et al.* (1) made the following three assumptions: (1) that glucose is catabolized to CO_2 by only two pathways, namely, Embden-Meyerhof glycolysis plus Krebs cycle and the direct oxidative pathway, (2) that, via the oxidative pathway, only the aldehyde carbon of glucose contributes to the CO_2, and (3) that, glycolytically, glucose yields 2 triose molecules whose carbons give rise to CO_2 in the same proportion as do the respective carbons of added lactate. On the basis of these assumptions, equations relating the ratio U to the recoveries of $C^{14}O_2$ from lactate-1-C^{14}, -2-C^{14}, and -3-C^{14} and the conclusion that a major portion of the CO_2 derived from glucose catabolism arose via the shunt were derived. A subsequent examination of these equations and calculations by Katz *et al.* (3) showed, however, that the amount of CO_2 produced from glucose by liver slices, via the direct oxida-

tive path, is small, and that the major portion of the CO_2 derived from glucose was formed glycolytically.

In the above considerations we have dealt only with the CO_2 derived from glucose. We must also consider *the fraction* of the glucose molecules catabolized by each pathway. Let us designate the *fraction of the CO_2 derived glycolytically* as E and the *fraction of the glucose catabolized glycolytically* as F. Both E and F can be expressed as functions of the $C^{14}O_2$ ratios U and W.

Expressions for E were derived previously (3) and are given in Equations 1 and 2.

$$E = \frac{R(6U - 1)}{U(6R - 1)} \tag{1}$$

$$E = \frac{6RW}{1 + W(6R - 1)} \tag{2}$$

where the parameter R is equal to $(c + d + e)/3e$ and c, d, and e are the yields of the $C^{14}O_2$ recovered from lactate-1-C^{14}, -2-C^{14}, and -3-C^{14}, respectively.

Expressions for F, the fraction of glucose catabolized glycolytically, can be derived if it is assumed that the carboxyl carbons of the phosphopyruvate derived from glucose are completely oxidized to CO_2. Then each mole of glucose oxidized glycolytically yields $2(c + d + e)/c$ moles of CO_2, and each mole of glucose oxidized via the shunt yields 1 mole of CO_2. Let n be the moles of glucose catabolized, nF moles be the fraction proceeding via glycolysis, and $n(1 - F)$ moles that proceeding via the shunt. Thus $((nF)\ 2(c + d + e))/c$ moles of CO_2 will be formed via glycolysis, and $n(1 - F)$ moles of CO_2 will be formed via the shunt. For simplicity, let us represent $2(c + d + e)/c$ by P. Employing the expressions for the specific activity of the CO_2 as derived for Equations 1 and 2, we obtain the following expressions for F as functions of U and W.

$$U = \frac{n\left[\frac{PF}{6} + \frac{1 - F}{6}\right]}{n\left[(1 - F) + \frac{PF}{6R}\right]}$$

whence

$$F = \frac{R(6U - 1)}{R(P - 1) + U(6R - P)} \tag{3}$$

$$W = \frac{n\left[\frac{PF}{6R}\right]}{n\left[\frac{PF}{6R} + (1 - F)\right]}$$

whence

$$F = \frac{6RW}{P(1 - W) + 6RW} \tag{4}$$

The relation between E and F can be expressed by a simple and useful equation. Let m moles of CO_2 be formed from glucose by both mechanisms, and let mE and $m(1 - E)$ moles be formed by glycolysis and the shunt, respectively. Since each mole of glucose yields P moles of CO_2 via glycolysis and 1 mole via the shunt, mE/P and $m(1 - E)/1$ moles of glucose, respectively, will be formed via these two paths. The fraction F

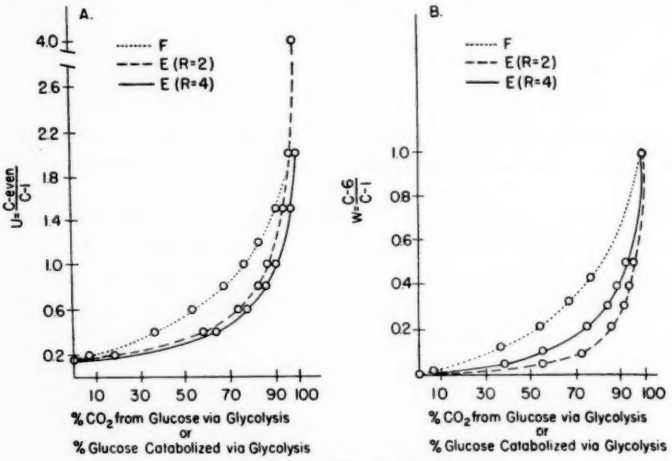


FIG. 1. Plot of Equations 1 to 4

can now be expressed by the equation

$$F = \frac{E/P}{\frac{E}{P} + (1 - E)} \tag{5}$$

Equations 1 to 4 are plotted in Fig. 1, with values of 2 and 4 for R and 3 for P. These values were selected because they represent the range of the experimentally determined values for R and P (see Tables II and III). Figs. 1, A and 1, B show that, when glycolysis predominates, a small increase in the extent of the direct oxidative pathway will cause a pronounced decrease in the values for the ratios U and W. The values for E, as calculated from Equation 1, are not appreciably altered by changes in the parameter R. As shown in Fig. 1, A, doubling of the value for R over the

whole range of the curve results in only a small decrease in the value of E. On the other hand, in the case of Equation 2 (Fig. 1, B), an increase in R

TABLE II

Oxidation of Lactate-C^{14} and Calculation of Values for R and P

Each vessel contained 50 μmoles of labeled *dl*-lactate and 50 μmoles of glucose. Other conditions as in Table I.

Rat No.	Label of lactate	Per cent of added C^{14} recovered as $C^{14}O_2$	$R = \frac{c+d+e}{3e}$	$P = \frac{2(c+d+e)}{c}$
6	C-1	47.8 (*c*)	2.8	2.8
	C-2	10.5 (*d*)		
	C-3	8.0 (*e*)		
7	C-1	48.5 (*c*)	3.2	2.7
	C-2	11.1 (*d*)		
	C-3	6.8 (*e*)		
8	C-1	48.2 (*c*)	3.0	2.8
	C-2	10.2 (*d*)		
	C-3	7.4 (*e*)		
8A*	C-1	40.3 (*c*)	2.6	2.8
	C-2	8.2 (*d*)		
	C-3	7.3 (*e*)		

* This rat was fasted for 24 hours prior to sacrifice.

TABLE III

Comparison of Values for Parameters R and P Obtained from Various Sources

Source	No. of rats	*R*		*P*	
		Range	Average	Range	Average
Table II	4	2.6–3.2	2.9	2.7–2.9	2.8
Bloom *et al.* (1)*	5	1.7–2.3	2.0	3.1–3.4	3.2
Felts " " (7)	3	2.2–3.0	2.6	3.1–3.4	3.2
Strisower†	4	3.2–3.8	3.4	3.2–3.8	3.3
Mean			2.7		3.1

* The incubation medium contained lactate, acetate, and gluconate.
† Unpublished experiments.

will cause an increase in the value of E, and, when the values for the ratio W are low, this effect becomes appreciable.

Values for R and P were calculated from experimental data obtained here and from those reported in the literature. These values are recorded in Tables II and III. The values for R ranged from 1.7 to 3.8, and those for P from 2.7 to 3.8. Although it is preferable to determine these param-

eters for each liver in which the glucose ratios are obtained, we have, for the purpose of subsequent calculations, chosen mean values of 2.7 and 3.1 (from Table III), respectively, for R and P.

Calculated values for the contribution of glycolysis to (1) the glucose-derived CO_2, *i.e.* E, and (2) the total glucose catabolized, *i.e.* F, are shown in the last two columns of Table I. When glucose was the sole substrate, the values for E ranged from 83 to 98 per cent. In other words, in some cases, practically all of the glucose-derived CO_2 arose via glycolysis. Under these same conditions, the values for F ranged from 61 to 94 per cent. When lactate, acetate, and gluconate were added to the medium in addition to the labeled glucose, the values for E and F were depressed and, as previously noted, the $C^{14}O_2$ yields from glucose-E-C^{14} and glucose-6-C^{14} were depressed to a much greater extent than those from glucose-1-C^{14}. From this it may appear that the additions to the incubation medium reduced the amount of glucose that was metabolized via glycolysis without affecting the operation of the direct oxidative pathway. It is also possible that glucose catabolism via glycolysis was not affected, but that the C^{14} activity was trapped in the large lactate pool.

It was also observed that the additions caused a doubling in the incorporation of labeled glucose into liver glycogen. Thus, in the presence of these additions, the percentages of the labeled glucoses recovered in glycogen were increased from 4 to 5 per cent to 9 to 10 per cent.

It is significant that variations in the experimental conditions greatly affect the values for the ratios U and W. Whereas, under our conditions, glucose seems to be catabolized primarily via the Embden-Meyerhof and Krebs cycle, other procedures of incubation or treatment of the rats may yield different patterns.

The most critical assumption involved in the derivation of the equations is that glucose carbons 2 to 6 do not contribute to CO_2. If, however, these carbons are oxidized to CO_2, the extent of the shunt will be greater than that calculated from Equations 1 to 5, and the discrepancy between the true and calculated values will be large for low values of the ratios U and W.

With regard to liver slices, this assumption is valid if the pentose formed via the shunt is completely reconverted to glucose and mixes with the glucose pool. In liver slices, in which only a small fraction of the added glucose is oxidized, the contribution of the pentose moiety to CO_2 by secondary oxidation will not greatly affect the magnitude of the calculated values for E and F. On the other hand, in tissues in which glucose oxidation is large, the resynthesized glucose formed via the shunt will be extensively metabolized; in this case CO_2 formation from carbons 2 to 6 will be appreciable. As has been pointed out in the case of rat mammary gland slices which metabolize glucose rapidly (13) or in systems in which this

recycling of metabolized glucose is extensive, Equations 1 to 5 cannot be used.

In the calculation of the fraction of the glucose catabolized via glycolysis, *F*, another assumption was made, namely, that carbons 3 and 4 of glucose are completely converted to CO_2. The validity of this assumption may be questioned, since C^{14} was always detected in lactate (see Fig. 2). The radioactivity incorporated into lactate was variable but quite low. Under other experimental conditions (14), however, the C^{14} incorporation into lactate may be larger, and in such cases the amount of glucose catabolized via the Embden-Meyerhof scheme will exceed that calculated by Equations 3 to 5. Thus when $C^{14}O_2$ production is used to estimate the relative contributions of these two pathways, *E* provides a more reliable index than does *F*.

Concerning Mechanism of Hexose Monophosphate Shunt

Chromatographic Patterns Observed in Metabolism of Glucose-1-C^{14}, -6-C^{14}, and -E-C^{14}

The products of the water-soluble fraction of the incubation mixture were examined by a chromatographic, radioautographic procedure similar to that reported for acetate-C^{14} (8). Typical chromatograms obtained in experiments with glucose-1-C^{14}, glucose-6-C^{14}, and glucose-E-C^{14} are shown in Fig. 2. About 90 per cent of the C^{14} in the water-soluble fraction was present as glucose, and the remaining C^{14} was accounted for by six or seven compounds, the most active of which was lactate. Glutamic acid, glutamine, and alanine were also identified. With the level of C^{14} used in these experiments, incorporation into any compound of as much as 0.5 per cent of the added C^{14} could be detected on the chromatogram. No significant differences in the chromatographic patterns were discernible when the experiments were carried out with labeled hexoses of high specific activity (Table IV). If the shunt did play a major rôle in glucose catabolism in liver, a considerable difference in the chromatographic patterns should have been detected.

The $C^{14}O_2$ ratios (*U* and *W*) and the chromatographic data can be explained on the basis of two possible mechanisms: (*a*) the exchange between C-1 of glucose and CO_2 and (*b*) the reconversion of the pentose moiety into glucose.

Exchange between C-1 of Glucose and CO_2—The reversibility of the decarboxylation of phosphogluconate has been demonstrated (15), and the possibility that this reaction accounts for some CO_2 fixation was suggested. To test this possibility, liver slices were incubated with C^{14}-labeled bicarbonate in the presence of unlabeled glucose or ribose. The C^{14}-glucose formed during the course of the incubation was isolated chromatographi-

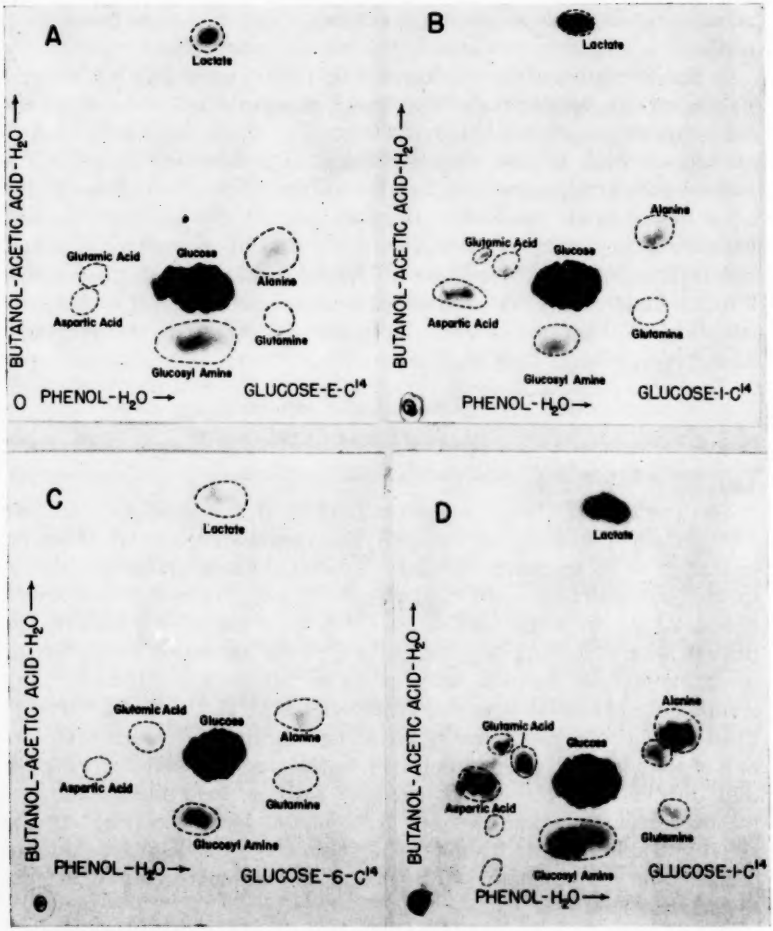


FIG. 2. Radioautographs of chromatograms obtained from rat liver slices incubated with variously labeled C^{14}-glucoses. In *A*, *B*, and *C*, chromatograms obtained from the liver of the same rat (Rat 20, Table I). The C^{14} activities of the labeled glucoses incubated were not identical, and the radioautographs were intentionally overexposed to show the less active compounds. In *D*, chromatogram obtained with a glucose sample of considerably higher specific activity than used in the other experiments.

cally and degraded. The results are shown in Table V. Most of the C^{14} was equally distributed between carbons 3 and 4, and no significant fixation of CO_2 into the aldehyde carbon occurred. Thus, the operation of an exchange mechanism in our system would appear to be excluded.

TABLE IV

Chromatographic Analysis of Water-Soluble Compounds Derived from Metabolism of Glucose-E-C^{14} and Glucose-1-C^{14} by Rat Liver Slices

The experimental conditions were the same as those described in Table I, except that about 0.1 mg. of a labeled glucose containing 10 μc. was used as substrate. The chromatographic procedure is described in the text. The values for the glucose-1-C^{14} experiment were determined from the chromatogram represented in Fig. 2, *D*.

Substrate	Distribution of C^{14} on chromatogram in							
	Glucose	Lactate	Alanine	Glutamate	Aspartate	Glucosylamine	Origin	Unidentified
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Glucose-E-C^{14}.....	93	1.2	0.8	0.9	0.5	1.2	1.0	1.4
Glucose-1-C^{14}.....	91	1.8	0.9	1.0	1.0	1.8	1.1	1.4

TABLE V

Distribution of C^{14} Recovered in Glucose Derived from Ribose-1-C^{14}

250 mg. of rat liver slices incubated with 3 μmoles of ribose-1-C^{14}. Conditions similar to those described in Table I. The additions of lactate, acetate, and gluconate were the same as those described in Table I.

Experiment No.	Substrate	Glucose carbon No.	Per cent of total C^{14} recovered in individual carbons
10 + 12*	$C^{14}O_2$ + unlabeled ribose	1	5
		2	4
		3	43
		4	43
		5	4
		6	1
10a + 12a*	Ribose-1-C^{14}	1	43
		2	4
		3	42
		4	5
		5	3
		6	3
10b + 12b*	Ribose-1-C^{14} + additions	1	44
		2	3
		3	40
		4	5
		5	6
		6	2
Rats 13–15*	Ribose-1-C^{14}	1	36
		2	5
		3	36
		4	11
		5	6
		6	6

* The glucose samples were pooled for purposes of degradation.

Ribose Metabolism by Rat Liver Slices—To test for the second mechanism, rat liver slices were incubated with ribose-1-C^{14}. It was catabolized to CO_2 and readily converted to glucose. As shown in Table VI, the $C^{14}O_2$ yields varied from 3 to 10 per cent when ribose served as sole substrate. The $C^{14}O_2$ recoveries were, however, markedly depressed by the addition of unlabeled lactate, acetate, and gluconate. When the aqueous extracts were examined chromatographically, the predominant C^{14}-labeled compound found was glucose, and very little C^{14} was detected in other compounds (Fig. 3). In one experiment (Rats 13, 14, and 15), all of the added ribose was utilized, but in Experiments 10a, 12a, 20, and 21, the utilization of the ribose did not proceed to this extent. In Experiments 10b and 12b,

TABLE VI

Oxidation of Ribose-1-C^{14} and Its Conversion to C^{14}-Glucose by Rat Liver Slices

For the description of the experiments see Table V and the text.

Rat No.	Flask	Additions to medium	Per cent of added C^{14} recovered as $C^{14}O_2$	Distribution of C^{14} on chromatogram		
				Glucose	Ribose	Others
				per cent	*per cent*	*per cent*
10	*a*	None	4.7	77	21	2
	b	+	1.1	65	34	1
11	*a*	None	8.1	79	20	1
	b	+	2.2	68	31	1
13		None	5.0	81	0	19
14		"	10.0	80	0	20
15		"	7.3	76	0	24
20		"	2.7	45	48	7
21		"	6.7	56	38	6

in addition to C^{14}-glucose and C^{14}-ribose, the only other labeled compound detected was lactate. In Experiments 10a, 12a, 20, and 21, some of the C^{14} was also found in alanine and glutamic acid. In the experiments with Rats 13, 14, and 15, in which no C^{14}-labeled ribose remained at the end of the incubation period, the C^{14} incorporation into compounds other than glucose amounted to about 20 per cent. Most of this 20 per cent was present in three unidentified compounds that were not observed in the other experiments, and the most prominent occupied a position overlapping that of urea.

Although the presence of ribokinase in microorganisms has been demonstrated (16), the existence of ribokinase has not hitherto been reported in animal tissues. Our findings, in conjunction with the well described conversion of pentose phosphate to hexose phosphate (17), suggest the existence of an active ribokinase in rat liver.

The mechanism of the conversion of a pentose phosphate to a hexose phosphate has been extensively studied at the enzyme level, and the results

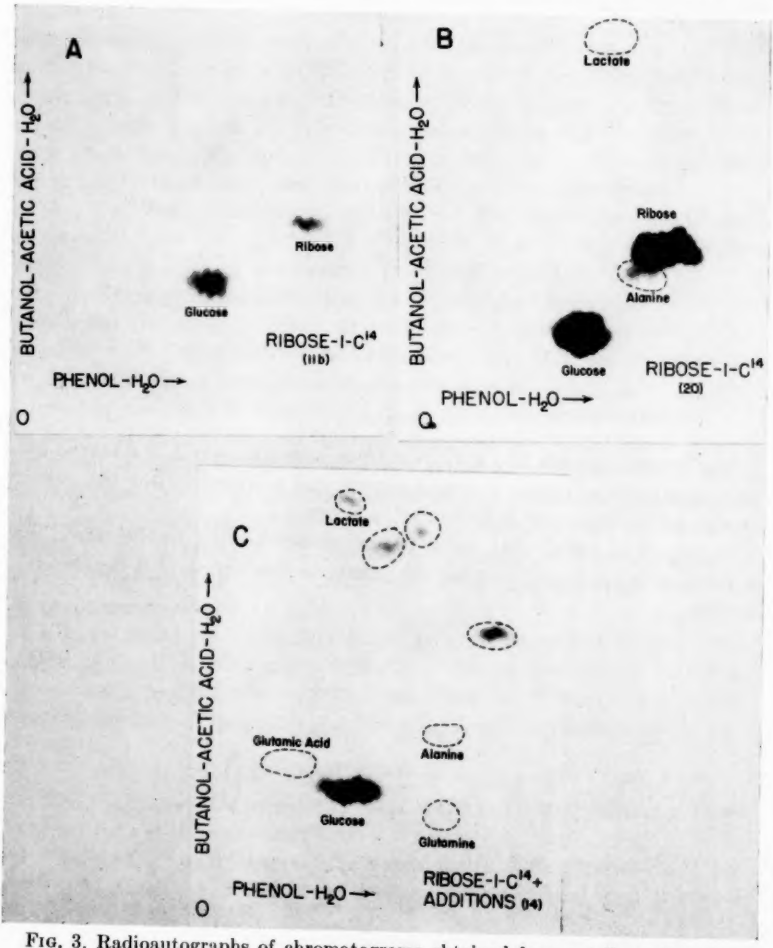
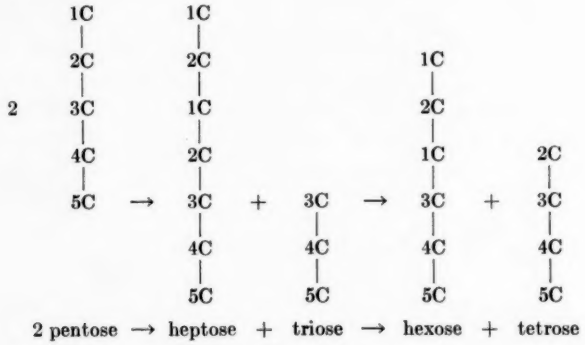


FIG. 3. Radioautographs of chromatograms obtained from rat liver slices incubated with ribose-1-C^{14}. The numbers in parentheses refer to the rat numbers (see Table VI). Chromatograms A and C were exposed for 1 week; Chromatogram B for 2 weeks.

are discussed in several reviews (16, 18–21). Thus far, studies on this conversion in rat liver slices have not appeared. The conversion of ribose to hexose can be formulated by two schemes. These schemes merely

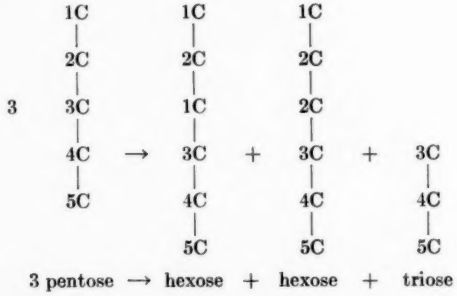
represent abbreviated reaction sequences elaborated by other workers. Only the carbon skeletons of the compounds involved are considered. The first scheme represents the conversion of 2 ribose molecules to 1 of glucose and 1 of tetrose (Scheme A).



SCHEME A

The tetrose can act as a glycolaldehyde acceptor, which is donated by a pentose molecule, giving rise to a hexose and a triose. Thus the over-all result can be expressed as presented in Scheme B.

It should be noted that, since the triose can also be resynthesized into glucose, Scheme B provides for the complete conversion of a pentose to a hexose.



SCHEME B

According to Schemes A and B, with ribose-1-C^{14} as substrate, only carbons 1 and 3 of the resulting glucose will be labeled. In the case of Scheme A, the ratio of C^{14} in carbon 1 of glucose to that in carbon 3 will be 1, and, in the case of Scheme B, the value for this ratio will be 2.

The results of the degradation of glucose formed from ribose-1-C^{14}, in

three experiments, are recorded in Table V. Most of the activity was found in carbons 1 and 3, and the observed ratio was about 1. Thus, our finding served to support Scheme A.

The low C^{14} activity of carbon 2 of glucose is of interest. If the 1,3-C^{14}-hexose phosphate formed from ribose-1-C^{14} is further metabolized via the shunt, hexose phosphate with C^{14} activity in carbon 2 will be formed by another turn of this cycle. The absence of significant activity in this carbon indicates that, in rat liver slices, the formation of free glucose from hexose phosphate, with subsequent mixing of such glucose with the general glucose pool, is much faster than the over-all operation of the shunt.

The $C^{14}O_2$ yield from glucose-1,3-C^{14} will be higher than that from glucose-1-C^{14}, and the observed $C^{14}O_2$ yields from ribose-1-C^{14} can be accounted for readily by the metabolism of such labeled glucose.

Our results suggest the formation of a tetrose which does not serve as an acceptor for the glycolaldehyde derived from pentose or heptose. This was unexpected, since the results of Racker *et al.* (22) and of Horecker *et al.* (17) imply that the tetrose can act as an acceptor.

Horecker *et al.* (17) studied the conversion of ribose-5-phosphate-1-C^{14} into hexose phosphate by a rat liver enzyme preparation and found 74 and 24 per cent of the C^{14} in glucose carbons 1 and 3, respectively. This result was considered to be in fair agreement with Scheme B. Experiments with ribose-5-phosphate-2,3-C^{14} also were in line with the operation of Scheme B; *i.e.*, 3 pentose $\rightarrow$ $2\frac{1}{2}$ hexose. The results obtained here with rat liver slices apparently are at variance with those obtained with cell-free preparations.

We have found no evidence for the accumulation of a tetrose, and, because of the high efficiency of the conversion of the pentose to hexose in many systems (23), it is suggested that the tetrose in some way is converted to glucose. This problem is under further investigation.

SUMMARY

1. The catabolism of glucose-1-C^{14} and -6-C^{14} and evenly labeled glucose-C^{14} of rat liver slices was studied.

2. The $C^{14}O_2$ yields observed in experiments with glucose-E-C^{14} and -1-C^{14} were about equal and about 3 times those observed with glucose-6-C^{14}.

3. The addition of a mixture of 50 μmoles each of lactate, acetate, and gluconate greatly depressed $C^{14}O_2$ formation from both glucose-E-C^{14} and -6-C^{14}, but had little effect on the $C^{14}O_2$ derived from the glucose-1-C^{14}.

4. Equations for calculating the extent of glucose metabolism in rat liver slices via the Embden-Meyerhof-Krebs route and the hexose monophosphate shunt are discussed. It was calculated, for rat liver slices,

that 83 to 98 per cent of glucose-derived CO_2 was formed glycolytically, and that 61 to 94 per cent of the total utilized glucose was catabolized glycolytically. When lactate, acetate, and gluconate (0.02 M) were added, a greater portion of the glucose catabolized apparently proceeded via the hexose monophosphate shunt.

5. The products of the metabolism of glucose-1-C^{14}, -6-C^{14}, and -E-C^{14} were examined by paper chromatography, and the metabolic patterns of all three labeled sugars were found to be similar. The significance of this finding is discussed.

6. Rat liver slices converted free ribose to glucose. It is therefore suggested that liver contains an active ribokinase. Glucose derived from ribose-1-C^{14} was isolated and degraded, and the C^{14} distribution in its carbons was determined. The C^{14} was equally distributed in carbons 1 and 3, with no significant incorporation in the other carbons. These results suggest that the pentose formed by the decarboxylation of 6-phosphogluconate is resynthesized into glucose by rat liver.

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